Host responses to respiratory infection in cystic fibrosis

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HOST RESPONSES TO RESPIRATORY INFECTION IN CYSTIC FIBROSIS

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To Andy

SUMMARY

Cystic Fibrosis (CF) is characterised by disrupted electrolyte and fluid transport because of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride (Cl^-) channel at epithelial mucosae. This condition is associated with a hyper-inflammatory pulmonary phenotype, which is exacerbated by chronic and repeated respiratory infections with bacteria such as *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Coupled with the effects of bacterial toxins, an excessive and uncontrolled immune response results in the production of copious amounts of mucopurulent sputum, leading to severe bronchiectasis and premature death in more than 90 % of patients. Much of the tissue destruction is host-derived, mediated by the potent neutrophil chemoattractant interleukin (IL)-8.

The aims of this study were to characterise IL-8 production and Cl^- channel activity by CF and non-CF airway epithelial cells, following stimulation with pharmacological or bacterial agonists, to identify any differences in host response that may be linked to the functional expression of CFTR. Cl^- channel activity was measured by fluorescence and radioisotopic labelling and the production of IL-8 and IL-10 was determined using ELISA. In addition, specific pharmacological inhibitors were employed to investigate the involvement of mitogen-activated protein (MAP) kinases in cytokine production.

It was shown that both *P. aeruginosa* and *B. cepacia* modulated Cl^- channel activity in the respiratory epithelium, although the effects did not appear to be CFTR-dependent. Furthermore, epithelial IL-8 production was increased via structural and secreted factors from both *P. aeruginosa* and *B. cepacia*, but these latter effects were antagonised by MAP kinase inhibitors, implying a role for MAP kinases in mediating secretion of this chemokine. In particular, p38 MAP kinase was required for IL-8 production by CF-cells, suggesting that this protein could provide a potential target for therapeutic intervention to ameliorate bacterial-derived tissue damage in the CF lung.

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ABBREVIATIONS

°C	degrees centrigrade
ΔF508	deletion of phenylalanine at residue 508
μCi	micro Curie
μg	microgram
μl	microlitre
μm	micrometer
ABC	ATP-binding cassette
ADP	adenosine diphosphate
ALP	anti-leukoprotease
AMPS	ammonium persulphate
APC	antigen-presenting cell
ASF	airway surface fluid
ASL	airway surface liquid
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CAA	casaminoacids
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cfu	colony forming units
CGD	Chronic Granulomatous Disease
cm	centimetre
cNOS	constitutive NOS
Da	dalton
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel

ERK	extracellular-signal regulated kinase
FCS	foetal calf serum
fMLP	formylated methinone-leucine-proline
g	gravity
g	gram
GDP	guanosine diphosphate
GI	gastrointestinal
GTP	guanosine triphosphate
h	hour
hBD	human beta-defensin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HRP	horse-radish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible NOS
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilo-dalton
L	litre
LB	Luria Bertani
LPS	lipopolysaccharide
M	molar
MAPK	mitogen-activated protein kinase
MBP	mannose-binding protein
MBq	mega becquerel
MEM	Minimal Essential Medium
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
moi	multiplicity of infection

MQAE	N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
MSD	membrane-spanning domain
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP
NBD	nucleotide-binding domain
NE	neutrophil elastase
NF IL-6	nuclear factor IL-6
NF_kB	nuclear factor kappa-B
NK	natural killer
nm	nanometre
nM	nanomolar
NO	nitric oxide
OD_x	optical density at wavelength, x
ORCC	outwardly-rectifying chloride channel
PBS	phosphate-buffered saline
PMSF	phenyl methyl sulphonyl fluoride
PRR	pattern recognition receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
RSV	Respiratory Syncytial Virus
SDS	sodium-dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMM	succinate minimal medium
SP-	surfactant protein
STI	soybean trypsin inhibitor
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylene diamine
TLR	Toll-like receptor
TM	transmembrane
TNF	tumour necrosis factor
U	units
UV	ultraviolet

V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1:

Introduction

Cystic fibrosis (CF) is the most frequent lethal genetic disorder in the Western world, affecting 1 in 2500 live births in Caucasian populations *(1)*. Relatively small numbers of other ethnic groups such as the Ashkenazi Jews and American Blacks are also affected *(2)*. First described by Dorothy Andersen in children with fibrocystic disease of the pancreas *(3)*, CF is a disorder of mucous membranes and the exocrine glands in which electrolyte and fluid transport are disturbed and this imbalance forms the basis for the 'sweat-test', which detects excessively high saline concentrations in the sweat and is diagnostic of CF *(4)*. Cystic fibrosis results from recessive mutations in the gene encoding a single chloride (Cl^-) channel expressed in apical epithelial membranes *(5;6)*. Hypertonic, viscid secretions obstruct the conducting tubes of a number of organs and the clinical consequences of CF range from meconium ileus, diabetes, pancreatitis and infertility to life-threatening lung dysfunction *(1)*.

Half a century ago the median survival age was 3-5 years, and malnutrition and staphylococcal pneumonia were the predominant causes of death in CF *(7;8)*. Gastrointestinal (GI) problems can be managed successfully now after the introduction of pancreatic enzyme therapy in the 1950s, although GI symptoms still cause significant morbidity. However, chronic and recurrent respiratory infections that are the hallmarks of CF cannot be eradicated and, following a massive and uncontrolled inflammatory response by the host, eventually lead to pulmonary failure and premature death in more than 90 % of CF patients *(4)*. Because of the intensive and multidisciplinary research effort during the past decade, the figure for life expectancy is approaching 40 years of age *(8)* and will increase as understanding and intervention strategies improve. CF is a particularly complex disease, understanding of which requires consideration of

three fundamental and inter-related elements, namely genetic mutation, inflammation and infection. This chapter provides a detailed analysis of the three components and examines how they contribute to the pathogenesis of CF.

1.1 The Genetics of CF.

1.1.1 Characterisation of the Disease.

In 1989 the autosomal gene responsible for CF was identified on the long arm of chromosome 7 and the encoded protein was named the 'cystic fibrosis transmembrane conductance regulator', or CFTR *(9-11)*. Since its discovery more than 850 recessive mutations have been reported within the gene (CF Genetic Analysis Consortium, *http://www.genet.sickkids.on.ca/cftr/*) and approximately 1 in every 25 people of North European extraction is heterozygous for a CF gene mutation *(2)*. Disease status is associated with genotype and covers a broad range of symptoms *(12)*. Patients with 'typical' CF, such as those with the most common genotype, *cftr* $^{\Delta F508}$ / $^{\Delta F508}$, display pancreatic insufficiency from an early age and develop severe lung pathology that ultimately is fatal *(1)*. At the opposite end of the clinical spectrum are patients with 'atypical' CF' who may not be diagnosed until adulthood because of less common symptoms *(13)*. These individuals suffer from mild or no respiratory or pancreatic disease *(12)*. Most CF patients fall somewhere between the two states, although a precise phenotypic profile for each genetic combination has not been delineated.

The clinical outcomes in the sweat gland and pancreas can be predicted from a known genotype but this 'genotype: phenotype correlation' is poor with respect

to the lungs, in part owing to the effects of so-called 'modifier genes' which can attenuate the severity of lung pathophysiology but which show different activity in different individuals *(14;15)*. It should be emphasised that siblings with CF may demonstrate different clinical progression since non-genetic factors can alter the outcome of this condition drastically *(16-18)*.

1.1.2 The carrier advantage in CF.

Since CF is such a devastating disease, it is reasonable to speculate why natural selection has allowed these mutations to remain in the gene pool. Three main hypotheses have been proposed on the basis of heterozygous advantage. *Escherichia coli* and *Vibrio cholerae* enterotoxins over-activate the CFTR Cl^- channel in the gut causing massive outpourings of watery stools and severe dehydration *(19;20)*. Such episodes are usually fatal in infants and in developing countries where hygiene and sanitation are poor. Indeed, diarrhoeal disease still poses the single greatest threat to public health worldwide, killing 3 – 4 million people each year *(20)*. Reduced expression of a functional CFTR Cl^- channel because of a heterozygous mutation would decrease the unwanted effects of these enterotoxins. Pier *et al.* have suggested that this selective pressure may be compounded by *Salmonella typhi*, the causative agent of typhoid fever, which enters gut epithelial cells by binding to CFTR *(21)*.

A third hypothesis states that non-CF carriers of ΔF508 are less likely to develop bronchial asthma *(22)*. This latter suggestion was proposed on the basis that diminished fluid movement across the respiratory epithelium would reduce the accumulation of intraluminal inflammatory mediators that usually would provoke an asthma attack *(22)*. However, this idea has been challenged *(23)*

because the incidence of asthma related deaths worldwide is relatively low. Understanding the different types of mutation in *cftr* will permit a more thorough assessment of these selective pressures and highlight potentially novel targets for therapeutic intervention. This understanding requires an appreciation of the biosynthetic processing of CFTR.

1.1.3 CFTR Biosynthesis.

In common with the majority of integral membrane proteins, CFTR is fed co-translationally into the endoplasmic reticulum (ER) lumen during synthesis at the ribosomes of the rough ER, glycosylated in the Golgi complex and inserted into the plasma membrane. Incorrectly folded CFTR is targeted for degradation via the ubiquitin-26S proteasome pathway *(24;25)*. CFTR is a complex, multi-domain protein with necessarily slow post-translational processing kinetics compared with the kinetics of degradation, and only 20-30 % of mature, functional CFTR is exported to the plasma membrane successfully *(26)*. This efficiency is reduced even further in CF.

1.1.4 Characterisation of CFTR mutations.

Missense, nonsense, frameshift and mRNA splice mutations in *cftr* have 5 broad molecular consequences, which are discussed in detail in several comprehensive references, for example *(27-29)*. These mutation classes are summarised in **Figure 1.1**.

1.1.5 The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR).

CFTR is known best as a cAMP-regulated Cl^- channel expressed at the apical epithelial membrane of most tissues *(30)*. However, a picture is evolving of

CFTR as a protein involved in a plethora of physiological processes within the cell and the relative contribution of each function of CFTR may need to be reassessed.

CFTR is composed of 1480 amino acids and has an approximate molecular mass of 170-kDa *(10)*. Two non-identical motifs, each containing a membrane-spanning domain (MSD) of 6 transmembrane (TM) α-helices, and a nucleotide binding domain (NBD) are joined to form CFTR by a large, highly charged cytoplasmic regulatory (R) domain *(10)* illustrated in **Figure 1.2.** CFTR interacts with other proteins **(Figure 1.3)**, which maintains its position at the membrane and mediates its regulation of other ion channels *(31-38;48;49)* .

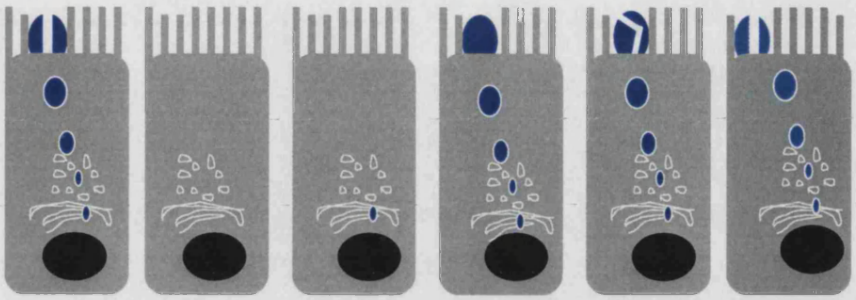
CFTR is a member of the ATP-binding cassette (ABC) superfamily of membrane transporters, which are found extensively in many organisms ranging from bacteria to mammals *(39)* although CFTR differs from other ABC transporters both structurally and functionally. Whilst the NBDs and 6-transmembrane-MSDs are defining features of this molecular class, the conjugal R-domain is unique to CFTR *(10;27)*. Coupling of the two motifs via the R-domain creates a low-conductance Cl^- channel, yet all other members of this gene superfamily assemble as ATP-driven membrane pumps *(39)*. Chloride can move freely in either direction through the pore, governed by activation of the channel and the electrochemical gradient across the membrane. In contrast, the movement of a substrate via its ATP-driven transporter usually is unidirectional and is limited stoichiometrically by ATP hydrolysis. CFTR is a multifunctional protein with different roles ascribed to distinct domains.

1.1.6 CFTR forms a functional chloride channel.

Unusually, gating of CFTR is dependent upon both ATP hydrolysis and phosphorylation by cAMP-dependent protein kinase A (PKA) *(40;41)*. It is accepted widely that the TMs assemble into the Cl^- channel pore and that positively charged residues such as Arg-352 **(Figure 1.2)** contribute to the anion-selectivity of CFTR *(42)*. Specific residues from TM domains in both MSDs are involved in channel function *(42)*.

1.1.7 Secondary functions of CFTR.

In addition to being an ion channel *per se*, CFTR regulates a number of other channels in the epithelium *(35-38)*. CFTR modulates cell proliferation and differentiation *(43)* and may regulate vesicular traffic *(44)*, as well as its potential role as an epithelial receptor for *P. aeruginosa (45)*. CFTR prevents the influx of sodium across the airway epithelium by down-regulating the amiloride-sensitive epithelial sodium channel, ENaC, through interactions with NBD-1 *(35)*, compounding the problem of dehydrated secretions at mucous membranes. Activated CFTR causes an efflux of ATP from the cell, which binds purinergic receptors on the luminal membrane face and stimulates a medium Cl^- conductance through outwardly rectifying chloride channels (ORCC) *(46)*. Therefore, absence or reduced expression of CFTR at the apical membrane can have secondary effects on transepithelial Cl^- conductance. These studies illuminate potential targets for pharmacological intervention in CF.



	Normal	Class I	Class II	Class III	Class IV	Class V
effect		**no synthesis**	**block in processing**	**block in regulation**	**altered conductance**	**reduced synthesis**
mutation type		Nonsense	Missense	Missense	Missense	Missense
example		**G542X**	**aa deletion ΔF508**	**G551D**	**R117H**	**A455E**

Figure 1.1 Classes of mutation affecting CFTR. Wild-type *CFTR* (blue) is transcribed in the nucleus (black) and processed in the Golgi and ER (white), where it is inserted into vesicle membranes and transported to the apical epithelial cell surface to form a functional chloride channel (far left). Numerous mutations affect one or more steps of the biosynthetic pathway resulting in failed expression of functional CFTR. For example, from left to right, mutation Classes I and II do not allow the production of any protein, whilst Class III produces correctly folded protein but dysregulation prevents its proper functioning as a Cl^- channel. Class IV mutations produce functional protein that reaches the membrane but cannot conduct Cl^- ions correctly, whilst Class V mutations affect the transcription or translation efficiency of CFTR so that fewer functional CFTR molecules are expressed at the apical membrane, compared with wild-type cells. Abbreviations: aa – amino acid. Adapted from ref. *(29)*.

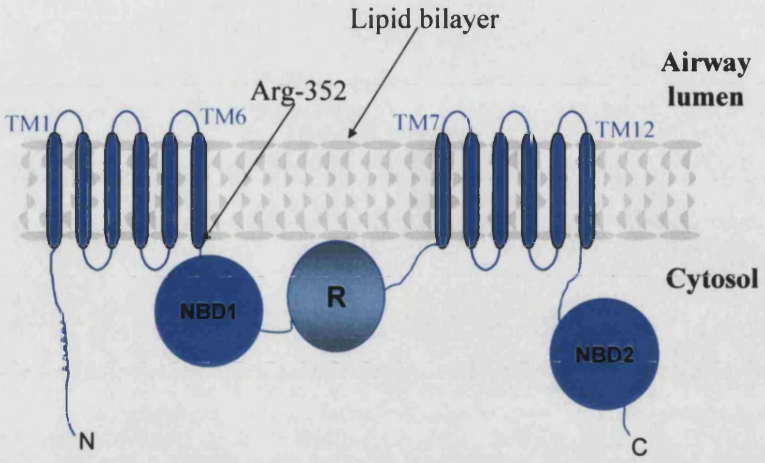


Figure 1.2 Membrane topology of CFTR. Two motifs, each comprising six TM regions and a NBD are joined by a highly charged regulatory (R) domain at the apical epithelial membrane. Arg-352 is a major determinant of charge selectivity of the channel. Adapted from ref.*(47)*

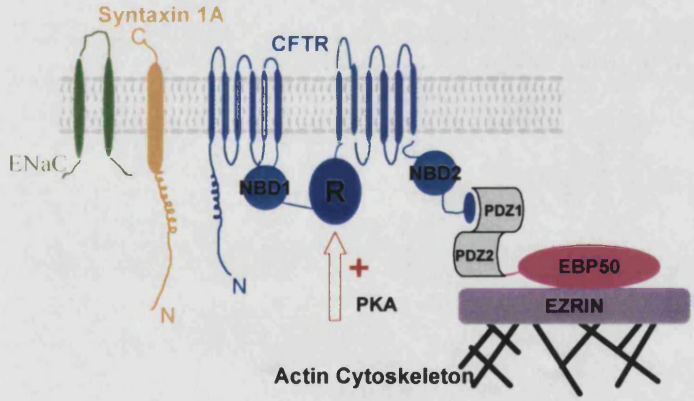


Figure 1.3 Interaction of CFTR with cellular components. Localization of CFTR to the apical membrane is via N-terminal interactions with syntaxin 1A *(48)*, and C-terminal interactions with the actin cytoskeleton, which are required for CFTR to function correctly *(49)* . EBP50 (ezrin-binding phosphoprotein 50) contains a PDZ domain t hat binds ezrin with great affinity, which in turn associates with cell scaffolding components such as actin (31).

1.2. The Host Response to Infection.

1.2.1 The immune response and inflammation in the CF lung.

Microbial products trigger an immune response in the local environment beginning with a rapid but non-specific reaction from components of the 'natural' (innate) immune system. Extensive networks of proinflammatory cytokines amplify the innate response and stimulate cells of the 'specific' (acquired / adaptive) immune system. Anti-inflammatory cytokines are released as the infection is cleared and the stimulus is removed, switching off the immune response when it is no longer required.

Resolution of the inflammatory response is essential since host-derived tissue damage is as detrimental as that instigated by a pathogen. Indeed, inappropriate immunological activity coupled with intractable bacterial infection results in the pathophysiological scenario in the CF lung. There is much *in vitro* evidence that CF pulmonary inflammation precedes infection *(50-52)*, including data from an uninfected ΔF508 mouse model *(53)* that demonstrated elevated respiratory levels of lymphocytes and inflammatory mediators. Contradictory findings state that inflammation occurs only after infection and increases relative to bacterial culture counts *(54)* but it is difficult to disregard the *in vivo* data. The inherent predisposition of the CF lung to inflammation likely is exacerbated by Respiratory Syncytial Virus (RSV), an important cause of inflammatory chest infections in very young children and particularly in those with CF *(55)*. One consequence of RSV infection seen in the alveolar cell line A549 *(56)* and human macrophages *(57)* was increased secretion of interleukin-8 (IL-8), findings that have important implications for CF. Depleted or

inactivated mechanisms of natural immunity could allow viruses to persist in the CF respiratory tract and establish an early infection-inflammation cycle that may encourage adapted bacterial pathogens to thrive.

Recurrent respiratory infections provoke an intensive and uncontrolled attack from non-clonal host factors. A functional adaptive response is activated but is ineffective in the CF pulmonary microenvironment. Cytotoxic molecules are released into the lung by the host with catastrophic effects on airway tissue so that ironically, the host itself causes much of the lung pathology associated with CF. The following section describes components of the immune response and how each may be affected by the absence or dysfunction of CFTR.

1.2.2 Physical barriers to infection at the epithelium.

The respiratory tract is lined with polarised epithelial tissue covered by a very thin layer (10-15 μm deep) of airway surface fluid (ASF; also known as airway surface liquid, ASL) *(58)* and a second layer of mucus that prevents bacterial adherence and traps inhaled particles in the airways. Both secretions contain a myriad of microbicidal agents. Thousands of cilia project from the apical epithelial surface and beat rhythmically into these secretions. However, disrupted transepithelial fluid and electrolyte transport experienced in CF has profound effects on the mucociliary clearance capacity of the lung, resulting in hyper-secretion of dehydrated viscid mucus that overwhelms the cilia *(59;60)*. The 'isotonic model' proposes that the CFTR defect reduces the volume of ASF that bathes the epithelium so that the cilia no longer can disengage the mucus bed to allow its propulsion *(61)*. An alternative and equally widely held view of the situation in the CF lung is termed the "hypotonic model". In this scenario

the volume of fluid bathing the lung remains the same as that in the normal lung, but the ionic composition of this ASF is altered, inactivating certain salt-sensitive proteins, as discussed later.

Another cardinal feature of CF is *P. aeruginosa* colonisation of the lung in 90 % of patients *(62)*. *P. aeruginosa* secretes pyocyanin and 1-hydroxyphenazine, both of which inhibit ciliary beating by disruption of cyclic nucleotide pathways *(63)*; and the lipopolysaccharide (LPS) component of *P. aeruginosa* outer membrane induces mucin glycoprotein synthesis via upregulated *MUC2* gene expression in the lung *(64)*. Another barrier to the establishment of microbes at the epithelial surface is desquamation of damaged or infected cells, a commonly used mechanism for preventing infection in the urinary tract. It has been proposed *(45;65)* that *P. aeruginosa* binds to CFTR and is taken up into the normal respiratory epithelial cell then sloughed-off for infected cells to be destroyed by phagocytosis. Hence, decreased pulmonary expression of CFTR in CF could allow the persistence of *P. aeruginosa* within the lung directly *(45;66)*, although there is by no means agreement on this point *(65;67;68)*. *P. aeruginosa* exacerbates and exploits many immunoregulatory features of the CF lung, discussed in the more specific context of bacterial infection. It is clear that the epithelium is not merely a physical barrier but that this structure actively produces and interacts with a multitude of host- and pathogen-derived molecules and cells and in so doing makes an important contribution during the immune response to infection.

1.2.3 Biochemical modulators of infection and inflammation.

Many natural biocides exist at the respiratory mucosa and yet in CF, pulmonary infection occurs repeatedly and chronically. Mutations in *cftr* reduce the expression of some of these biochemical factors; whilst the functional integrity of other components may be disrupted by the abnormal tonicity of CF respiratory secretions *(69-71)*. Both salt-insensitive and salt-sensitive agents are discussed to highlight how a failure of each mechanism potentially could affect CF respiratory immunology.

1.2.3.1 Complement

Complement-mediated elimination of potential pathogens is achieved by opsonisation, target cell lysis, chemotaxis, and immune complex removal, which have been ascribed to different complement factors *(72)*. The intermediate C5a has inflammatory effects that include degranulation of basophils and mast cells; and increased vascular permeability, allowing extravasation of neutrophils and monocytes to the site of infection. The C5a fragment and its receptor (C5aR) were examined as potential culprits for the ineffectiveness of CF innate immunity and a local deficiency in C5a and its receptor in CF airways has been postulated *(73)*. Knockout mice lacking C5aR were shown to suffer more severe *P. aeruginosa* infection than did their C5aR-competent littermates *(74)* despite normal trafficking and infiltration of neutrophils. Precisely why depletion of the C5a system should impair the clearance of *P. aeruginosa* from the lung is unclear. Murine models also were used to investigate the properties of collectins, another group of antimicrobial mediators that is affected in CF.

1.2.3.2 Collectins.

Collectins include the surfactant proteins, SP-A and SP-D, and mannose-binding protein (MBP), which mediate host-parasite interactions including opsonisation, bacterial aggregation and enhanced uptake of intracellular pathogens *(75)*. Expression of SP-A and SP-D is reduced in some CF patients *(76)* which may lead to reduced clearance of both *P. aeruginosa (77)* and *Staphylococcus aureus (75)*.

1.2.3.3 Nitric oxide

Nitric oxide (NO) regulates distinct cellular processes depending on its localisation and concentration, as discussed in several reviews *(78;79)*. NO is synthesised from L-arginine by nitric oxide synthase (NOS) which exists in at least three different isoforms. The calcium-dependent isoform is expressed constitutively (cNOS) and the inducible isoform (iNOS) is expressed during inflammation *(80)*. Usually, calcium-dependent cNOS produces NO at picomolar concentrations, which regulate vasorelaxation tightly. However, during endotoxic shock, the iNOS-derived levels of NO increase in response to high levels of bacterial LPS with the result that relaxation of smooth muscle and blood vessels becomes uncontrolled *(81)*. Nanomolar amounts of NO are generated by iNOS in cells of the immune system providing an important innate defence mechanism against pathogens *(82;83)*. Very recently, NO has been shown to potentiate killing of *Burkholderia cepacia* by hydrogen peroxide *(84)* and to a lesser extent, killing by superoxide. Oxidation of nitrogen species by superoxide generates peroxynitrite ($ONOO^-$), a potent bactericidal molecule that attacks prokaryotic copper- and iron-containing proteins to release free Cu^{2+} and Fe^{2+} and highly toxic, oxidising hydroxyl and superoxide radicals *(85)*. It has

been suggested that there is a positive correlation between NO levels and bronchiectasis, which is probably mediated by IL-1β and TNF-α. Exaggerated NO production is observed in asthma but not in CF, indeed some research shows iNOS expression to be reduced considerably in cells displaying mutant CFTR *(86)*. Other published data suggest that total exhaled oxidised nitrogen species are elevated in CF *(87)*. NO activates cGMP and by doing so NO can regulate respiratory transepithelial Cl^- currents *(88)*, which is also of considerable relevance to CF.

The molecular basis for reduced expression of NOS, C5aR and surfactant in the CF lung has not been explained satisfactorily but several researchers have proposed that the impotence of some other antimicrobial agents within the CF lung, such as defensins, may be related to disturbed tonicity in CF pulmonary secretions.

1.2.3.4 Defensins

Defensins belong to a large and highly conserved family of small (3 – 6-kDa) endogenous cationic antimicrobial peptides *(89)*. Many eukaryotic species including plants and insects have been shown to express defensin homologues (see reference *(90)* for a recent review) and this phylogenetic conservation highlights the importance of such molecules in innate immunity. Peptide antibiotics have variable broad-spectrum activity against bacteria, fungi and some enveloped viruses *(91)*. β-defensins are synthesised locally and continuously by mammalian epithelial cells *(71;92-95)* and their expression may be upregulated by a local inflammatory environment. Type-1 human beta defensin, hBD-1, is expressed constitutively at tissue-specific levels in many

sites including renal, intestinal and respiratory epithelia *(71;92;96)*. In contrast, Type-2 beta defensins including hBD-2 *(94)* and mouse β defensin 3 *(97)* are expressed constitutively at much lower levels but may be induced by microorganisms and their products, as well as by inflammatory mediators *(95)*. Reported levels of defensins may differ slightly from one study to another, but it is clear that these molecules play a major role in mammalian epithelial immunology.

Cationic defensins kill bacteria by disrupting high affinity glycolipid-ionic electrostatic forces in the prokaryotic membrane and creating ion-permeable pores *(98)*. Electrostatic interactions with LPS confer anti-endotoxic properties on defensins *(99)*, potentiating the anti-inflammatory effects of these peptides. Defensins have been implicated in CF as potentially major culprits of failed innate respiratory defence. Seminal research in 1996 at the University of Iowa, USA demonstrated that ASF from non-CF airways inhibited the growth of clinically important CF pathogens but that hypertonic respiratory secretions from CF airways did not *(69)*. This bacterial inhibition was shown to be reversibly salt-sensitive. Within a short time, another research group confirmed suspicions that hBD-1 was responsible for at least some of the salt-sensitive antimicrobial activity of ASF *(71)* and more recently, hBD-2 has been implicated as an additional candidate for impotence against *P. aeruginosa* infection in CF *(93;100)*. These intriguing observations had identified a major and plausible link between the genetic defect and recurrent pulmonary infection in CF, and suggested that clinical restoration of defensin activity could be advantageous to CF patients suffering from *S. aureus* or *P. aeruginosa* infections.

Chronic Granulomatous Disease (CGD) patients lack NADPH reductase so these individuals rely on non-oxidative antimicrobial mechanism, such as defensins, to kill invading bacteria *(101)*. Interestingly, CGD patients as well as CF sufferers, become colonised with *B. cepacia (101)*, suggesting that *B. cepacia* is resistant to defensin-mediated killing. This resistance was confirmed very recently *(102)* so that restoration of defensin activity will have little benefit for CF patients colonised with *B. cepacia.*

1.2.3.5 Cytokines co-ordinate the immune response

Cytokines are peptide hormones that range up to 40-kDa in size and are secreted by many cell types, regulating further cytokine production, cell proliferation or apoptosis. For example, macrophages produce IL-1β, TNF-α and IL-8 in response to bacterial antigens such as LPS. IL-1β and TNF-α are potent early-response cytokines, sharing many effects and acting on a range of cell types including neutrophils, epithelial cells and the vascular endothelium to attract neutrophils to the site of infection via IL-8 signalling, discussed later. In addition, IL-1β and TNF-α act in both a paracrine and an autocrine fashion on macrophages and this activity serves to magnify and reinforce the inflammatory response. Unusually high systemic levels of IL-1β and TNF-α resulting from chronic activation of the immune system may exacerbate cachexia, a common symptom of CF that stems from pancreatic insufficiency and other gastrointestinal complications *(103;104)*. Whilst high concentrations of TNF-α can induce apoptosis in some cell types *(105)*, no lethal effects of IL-1β have been observed. Furthermore, TNF-α is not produced by epithelial or endothelial cells, yet both tissues synthesise IL-1β *(106)*. Thus, despite having

some targets and effects in common, these cytokines display some important differences so that neither IL-1β nor TNF-α is functionally redundant. Notably, *B. cepacia* induces human monocytes to increase their production of TNF-α *(62)*, which could add significantly to CF pulmonary inflammation.

The anti-inflammatory cytokine IL-10 is produced mainly by type-2 helper T (T_H2) lymphocytes and is an important molecule in the resolution of the immune response by reducing the inflammatory effects of macrophage-derived cytokines induced by LPS *(107)*. Some epithelial cells also secrete IL-10 but the expression of this cytokine is reduced in CF in both epithelial and lymphocyte cells *(103;108-110)* and consequently the activity of IL-1β and TNF-α from alveolar macrophages is not regulated. Decreased secretion of the anti-inflammatory IL-10 may be as important as increased pro-inflammatory mediator production in creating an uncontrolled inflammatory environment at CF respiratory epithelia.

Some cytokines, such as IL-8, induce directional chemotaxis of leukocytes, earning them the name chemokines (a contraction of *chemo*tactic cyto*kines*). Chemokines constitute a group of 8 – 10-kDa molecules that act via subsets of G protein-coupled receptors on a multitude of different cell types with inflammatory results (see *(111;112)* for recent reviews). Neutrophil elastase and IL-1β both induce IL-8 release from respiratory epithelial cells and from neutrophils *(113;114)*, as does neutrophil-derived IL-8 *(115)*, establishing a localised cycle of inflammation. Several products of *P. aeruginosa* and *B. cepacia* elicit IL-8 from epithelial cells and peripheral blood monocytes *(116;117)* although the underlying molecular mechanisms have not been

delineated. Surprisingly, recent work by Schwiebert *et al. (118)* challenges the notion that upregulated IL-8 expression correlates with defective CFTR expression but despite their claim that differences in IL-8 production between CF and non-CF tissues are insignificant, data presented in this paper show greater basal production of IL-8 in some CF cell lines so that their dismissal of CFTR-dependent IL-8 expression should be accepted with caution. Even more intriguing was the observation by Massengale *et al. (119)* that in fact IL-8 production is decreased in CF cells compared with those that expressed wild type CFTR. Nevertheless, it is clear that IL-8 and other cytokines are important molecules that synchronise the biochemical and cellular defence systems in the immune response to infection.

1.2.4 Cell-mediated pulmonary immunity.

1.2.4.1 Pulmonary macrophages.

Respiratory macrophages are of particular importance as a source of pro-inflammatory mediators, as a link between lung and systemic responses, and in the removal of immune complexes from the CF lung *(120)*. Macrophages constitute the major surveillance cells in host defence, patrolling the epithelial mucosae for foreign molecules, which they bind and remove by phagocytosis. Stimulated macrophages releases IL-1β and TNF-α that recruit and activate numerous target cells including neutrophils and the epithelium, which express cell adhesion molecules and secrete additional inflammatory signals. Despite the eventual activation of specific lymphocytes, CF pathogens are not eliminated and the innate responses become chronic with severe pathophysiological consequences. Under normal circumstances the macrophage would initiate wound healing, as the infection is resolved. In CF, pulmonary

infection is not cleared so these processes are activated chronically and thick scars form at sites of host-derived pulmonary damage, caused mainly by neutrophils.

1.2.4.2 Neutrophils

By far the most abundant leukocyte in the CF lung is the neutrophil *(120)*, or polymorphonuclear leukocyte (PMN), and its most potent stimulus is IL-8 *(121)*. IL-8 is produced by the epithelium, the endothelium and macrophages in response to macrophage-derived IL-1β and TNF-α *(113-115)*. Combinations of IL-1β, TNF-α and IL-8 up-regulate the surface expression of adhesion molecules on the neutrophil, the endothelium and tissue through to the respiratory epithelial mucosa *(120;122)* in order to facilitate this recruitment of neutrophils by diapedesis.

Activated neutrophils possess a variety of bactericidal mechanisms such as digestive enzymes, antimicrobial peptides, and the synthesis of reactive oxygen and nitrogen species (ROS and RNS, respectively), which are compartmentalised in neutrophil granulocytes to prevent host-cell damage. In CF, however, the activated neutrophil is unable to engulf target bacterial cells because of alginate production and the formation of bacterial microcolonies termed biofilms *(7)*. Instead, the neutrophils empty their lysosomal contents including anionic radicals and elastase into the extracellular milieu where these molecules attack the lung epithelium. Elastin provides alveoli with their essential plasticity so the host secretes small amounts of anti-leukoprotease (ALP) *(123)* and α_1-antitrypsin *(124)* to counteract spurious elastase leakage. However, the combined elastase derived both from neutrophils and

P. aeruginosa overwhelms these inhibitory molecules *(125)*. Thin, flexible lung tissue is replaced by thickened fibrotic material so that gaseous exchange across the CF lung is impaired severely. Neutrophil elastase (NE) elicits further IL-8 release from neutrophils and from respiratory cells, as demonstrated in a *cftr* $^{\Delta F508/\Delta F508}$ cell line *(126)*, establishing a local autocrine amplification cycle of inflammation.

1.2.4.3 Role of adaptive cellular immunity in CF.

The specific arm of host defence is provided by T lymphocytes and B lymphocytes, and functions normally in CF *(120)*. Activated B cells synthesise and release a variety of Ig isotypes including IgA, which is important in eliminating respiratory infections. In support of this hypothesis, both *Haemophilus influenzae (127)* and *P. aeruginosa (128)* produce IgA-specific proteases that degrade this immunoglobulin isotype as a mechanism for averting the host response.

Two major populations of T cells are defined according to whether they express CD4 (helper T, T_H cells) or CD8 (cytolytic T, T_C cells). T_H2 cells secrete IL-4, IL-5, IL-10 and IL-13 and are associated particularly with an allergic or humoral (Ig-mediated) immune response against extracellular pathogens *(129)*. In contrast, the predominant T_H1 cell-derived cytokines are IFN-γ and IL-2 and this lymphocyte sub-population assists $CD8^+$ T cells eliminate intracellular infection *(129)*. IFN-γ also activates B cells (which produce opsonising immunoglobulin) and macrophages to enhance phagocytosis of infecting pathogens. Bronchial epithelial cells stimulated with IFN-γ produce chemokines that signal through the CXCR3 molecule found predominantly on

T_H1 cells thus amplifying the T_H1 response *(130)*. Neither IL-1β nor TNF-α induced the production of these chemokines from the bronchial cells, but instead elicited the detrimental production of IL-8 *(130)*. Not only is IL-8 a pivotal mediator of excessive neutrophil activity in CF, but also this chemokine drives T_H2-mediated production of antibody against extracellular organisms that is ineffective in CF *(131)*.

B. cepacia has been reported to invade *(132)* and replicate in activated macrophages *(133)*, as well as replicating in epithelial cells *(134)*. It is possible therefore that T_H1 lymphocytes would be more effective than T_H2 cells against CF infections with *B. cepacia* and recent data provided by Sauty *et al.* demonstrate that this could be achieved by stimulating epithelial cells with IFN-γ *(130)*. Another study by Moser *et al. (135)* examined CF patients with chronic *P. aeruginosa* infection and showed that these patients exhibited T_H2 responses most frequently, and that this type of response correlated with poor lung function compared with those patients exhibiting T_H1 responses. Thus, driving the immune system towards T_H1 and away from T_H2 type responses could benefit chronically infected CF patients.

1.2.5 Co-ordination of the response via intracellular signalling.

Cells respond to environmental stimuli, such as the presence of bacterial products or pharmacological agents, in a bewildering variety of ways that may be mediated directly, for example via protein phosphorylation, or indirectly via second messengers. Extracellular information is relayed to the nucleus via intracellular signalling cascades that begin in general with a ligand-receptor interaction at the membrane. Many receptor types and signalling pathways have

been described, but discussion here is restricted to interactions that regulate two distinct and important airway epithelial cell responses, namely activation of the CFTR Cl^- channel and production of inflammatory mediators.

1.2.5.1 Activation of CFTR

Pharmacological mediators that activate the CFTR Cl^- channel operate by means of the second messenger, cAMP, as depicted in **Figure 1.4**, allowing rapid and transient control of Cl^- efflux via CFTR. In contrast, some cellular effects display much slower reaction kinetics and occur over periods of minutes or hours. For example, transcription of inflammatory components mediated by a cohort of kinase and phosphatase molecules in response to IL-1β and TNF-α can take several hours.

1.2.5.2 Signalling via IL-1β and TNF-α.

These pathways have been relatively well characterised and are depicted in **Figure 1.5.**

1.2.5.3 MAP kinase signalling cascades.

Mammalian cells possess three main families of MAP kinases, namely extracellular signal regulated kinase (ERK), and the stress-activated protein kinases (SAPK) p38 MAP kinase and c-Jun N-terminal kinase (JNK), which regulate development, growth, protection against stress, the immune response and prevention of damage to DNA *(136-139)*. MAP kinase molecules respond synergistically to distinct extracellular cues and their complex pathways display a degree of overlap and redundancy, whilst allowing for amplification of the original signal. A bewildering amount of information has been accumulated in

recent years regarding MAP kinase signal transduction, contributing significantly to the complexity of the subject, and the literature reveals some apparent contradictions regarding MAP kinase activity and physiological consequences that may be cell- or tissue-specific. For this reason, a much-simplified overview is presented here describing the salient features of the three MAP kinase signalling cascades **(Figure 1.6)**, followed by discussion of some common downstream effects that culminate in the activation of gene transcription in the nucleus. An exhaustive and detailed discussion is outwith the scope of this work, but more detail is provided by several recent reviews *(137-140)*.

1.2.5.4 Signalling through ERK.

ERK is probably the most well studied MAP kinase and it is activated by endotoxin, IL-1β and certain growth factors like epidermal growth factor (EGF) *(136;141)*. ERK exists as 44-kDa (ERK-1) and 42-kDa (ERK-2) isoforms and a specific inhibitor of the ERK cascade, PD-098059 *(142)*, is invaluable in studying the cell-specific activation of ERK by numerous stimuli. As well as their effects on ERK signalling, IL-1β and endotoxin also may activate the SAPK pathways of p38 and JNK, which are less well characterised than the ERK pathway, although some overlap exists between these parallel cascades.

1.2.5.5 Signalling through Stress-Activated Protein Kinases (SAPK).

JNK and p38 share approximately 50 % sequence homology with ERK *(138)* and they are activated preferentially by cellular stresses and have diverse physiological roles in processes that include the immune response, cell proliferation and apoptosis *(137-139)*. Also known as reactivating kinase, p38

was discovered in 1994 as a kinase that responded specifically to endotoxin and hyperosmolarity *(143)* but whilst osmoreceptors have been identified in yeast cells, analogous molecules have yet to be found in mammalian systems *(137)*. p38 is found in evolutionarily diverse eukaryotic cells ranging from yeast to mammalian tissue, and much of the information regarding p38 has come from studies in *Drosophila.* Four mammalian p38 isoforms have been found that share 60-75 % sequence homology and show different substrate specificities *in vitro (138)*. As many as twelve isoforms of JNK exist by virtue of alternative splicing of the three JNK genes (α,β and γ) *(137)*, but all are approximately 54-kDa in size. The major function of JNK appears to be in stress-induced cell death, either by apoptosis or necrosis *(137)*, but the lack of a specific functional inhibitor of the JNK pathway impedes detailed study of this cascade. In addition, JNK activates NFκB, whilst this transcription factor is inhibited by p38 kinase *(138)*. The serine / threonine kinases MAPKAPK2 and MAPKAPK3 serve as additional substrates for p38 and these proteins phosphorylate and activate a number of heat-shock proteins within the cell *(138)*. A final point of interest regarding the physiological functions of the MAP kinases is their involvement in directing the immune response during infection. Whilst ERK cascades favour T_H2-type responses, the SAPKs promote T_H1-type responses *(139)* and this feature may be of clinical relevance in CF.

1.2.5.6 Signalling through NFκB.

The sequence of events leading to NFκB activation, including proteins involved in phosphorylation and activation reactions, are shown in **Figures 1.6 and 1.7.**

Upon cellular activation, a MAP3K is activated and phosphorylates the IKK (IκB kinase) complex. Possible MAP3K candidates include MEKK-1 of the JNK cascade *(144)*, although MEKK-1 was found not to be necessary for cytokine-induced JNK or NFκB activation in murine macrophages and fibroblasts *(145)*. Mitogenic stimuli that activate the Raf pathway (upstream of ERK) can activate NFκB independently of ERK, diverting via MEKK1 *(146)*. Cytokine induction of IKK activity may be mediated by NIK *(147)*, another MAP3K. The IKK complex consists of IKKα (IKK-1) and IKKβ (IKK-2) catalytic subunits and an IKKγ regulatory subunit *(148)*. A putative kinase implicated in the phosphorylation of IκBα is $p90^{rsk1}$ *(149)* a serine threonine kinase that lies downstream of the ERK MAP kinase cascade. The IKK complex phosphorylates IκBα at Ser32 and Ser36 *(150)* or IκBβ at Ser 19 and Ser23 *(151)*, the IκB protein is ubiquitinated and degraded via the 26S proteasome pathway *(150)*. Both Ser32 and Ser36 residues must be phosphorylated to effect ubiquitination and subsequent proteolysis, since mutation of either one or both of these residues prevents the degradation of IκBα *(150;152)*.

In quiescent cells, IκB binds (and inhibits) via its ankyrin repeat domain (ARD) to NFκB *(153)*. The mammalian transcription factor NFκB is central to the regulation of genes involved in numerous cellular responses, and in particular inflammation *(154)*. NFκB is a dimeric protein consisting of two Rel-related protein subunits: a 65-kDa subunit that contains the Rel-homology domain (RHD) and a 50-kDa subunit *(154;155)*. Following activation of the IKK complex and degradation of IκB, the NLS on NFκB is exposed and NFκB

translocates rapidly into the nucleus *(155)*, whereupon it binds to the DNA sequence 5'-GGGPuNNPyPyCC-3' *(156)* present upstream of the promoter in a multitude of genes, to initiate their transcription. Gene transcription also requires phosphorylation of the NFκB p65 subunit *(157)* and that this moiety binds the phosphorylated TATA-binding protein (TBP) *(156)*. The IκB family members display different affinities for NFκB and different modes of action. For example, IκBα is degraded rapidly but is resynthesised within an hour, under the control of NFκB *(158)*, and re-complexes with NFκB to terminate gene transcription. However, IκBβ transcription is not controlled by NFκB and when resynthesised, IκBβ is either unphosphorylated or hypophosphorylated so that it does not mask the NFκB NLS or DNA-binding sites *(151;154)*. As such, NFκB cannot bind and be inhibited by IκBα so that IκBβ-mediated NFκB stimulation is for a protracted period (>20 h) *(154)*. These differences allow fine-tuning of gene transcription of NFκB. In CF bronchial epithelial cells, NFκB activation is mediated primarily by IκBβ, resulting in prolonged NFκB activity and increased production of inflammatory mediators *(159)*.

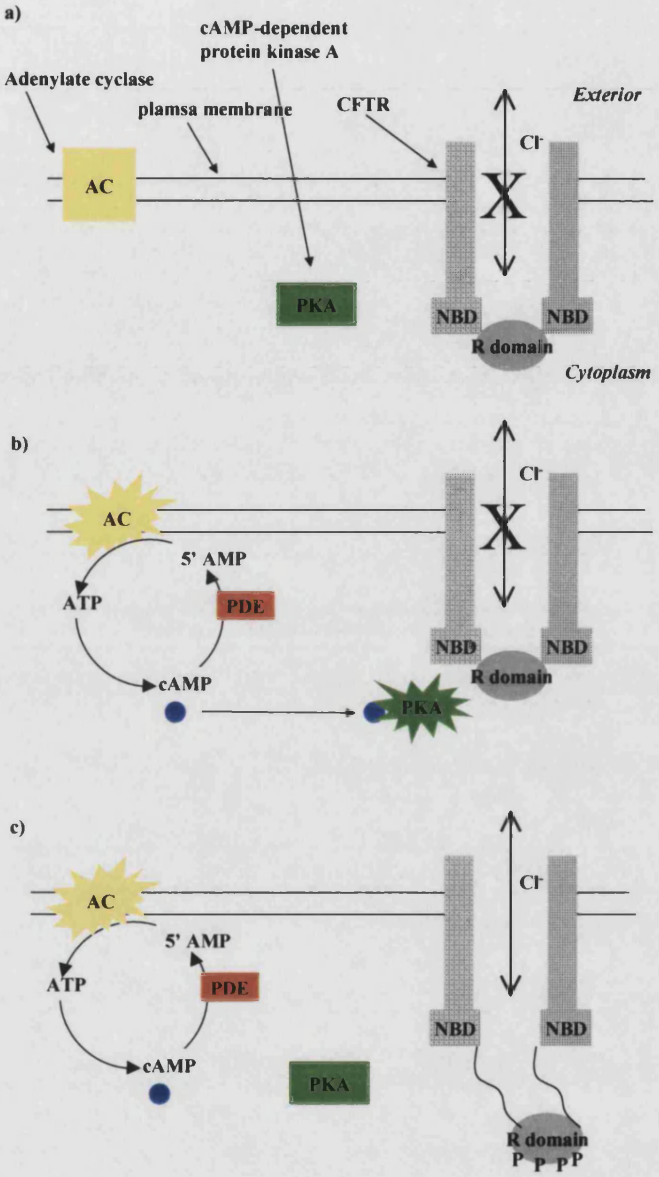


Figure 1.4 CFTR activation by cAMP. In the resting state, the CFTR Cl^- channel is blocked sterically by its R-domain (**a**). Activated adenylate cyclase (AC) converts ATP to cAMP (**b**) and this second messenger molecule amplifies the response since each molecule of adenylate cyclase produces many cAMP molecules during the time that $G_{s\alpha}$-GTP remains bound. cAMP-dependent protein kinase (protein kinase A, PKA) then phosphorylates numerous sites in the R-domain of CFTR, inducing a conformational change such that the R-domain assumes a new position within the cytoplasm, thus unblocking the channel (**c**). The effects of cAMP are transient since it is converted back to 5' AMP by phosphodiesterase (PDE) enzymes.

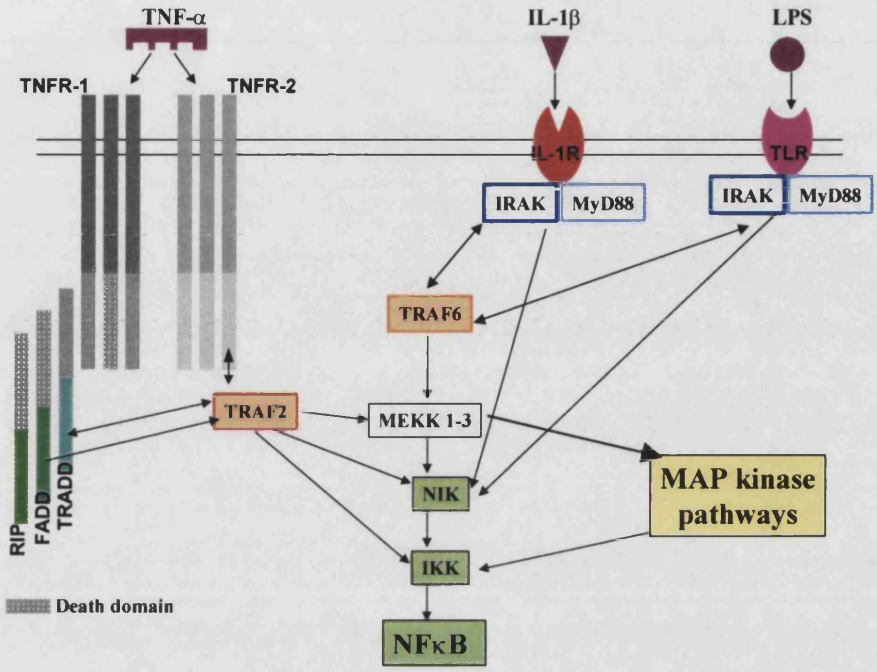


Figure 1.5 Signalling via TNF-α and IL- 1β. TNF-α binds to its receptor (TNF-R1 or TNF-R2) and recruits the protein TRAF- 2 (TNF-receptor associated factor) directly or via the 'death-domain'-containing molecules, FADD (Fas- associated death domain protein) and TRADD (TNF receptor-associated death domain) and receptor interacting protein (RIP), which associate with the cytoplasmic portion of the TNF receptor. The death domain consists of approximately 80 amino acids and this binding site also mediates TNF-induced apoptosis. IL- 1 receptor activation recruits the IL- 1 receptor-associated kinase (IRAK) and TRAF- 6, and the IL-1β and TNF-α pathways converge on the NFκB inducing kinase (NIK) molecule, activating NFκ B as described below. Furthermore, TRAF proteins may activate another kinase called MEKK-1, which forms part of the mitogen activated protein kinase (MAP kinase) cascade and ultimately may activate NFκB as well. LPS signalling via Toll-like receptor (TLR) shares many features of IL-1β signalling, including accessory proteins such as IRAK and MyD88.

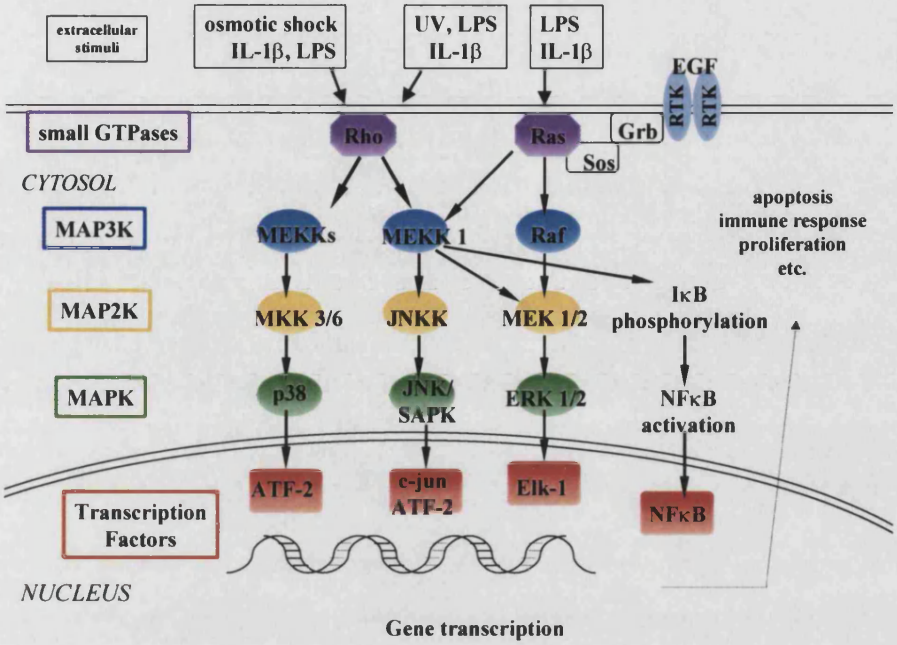


Figure 1.6 MAP kinase cascades. Binding of a ligand, such as EGF, to its receptor tyrosine kinase (RTK) induces dimerisation of the receptor, followed by autophosphorylation of tyrosine residues in the cytoplasmic tail of the receptor molecule. An adaptor protein, Grb2, associates with the phosphorylated tyrosine residues in the receptor tail via an SH2 domain, whilst the SH3 domain of Grb2 binds a cytosolic protein, Sos (son of sevenless), a GTP-exchange factor (GEF) and localises it to the plasma membrane. Sos binds and activates Ras, a small GTP binding protein, which exchanges GDP for GTP and in doing so becomes activated. Ras- GTP associates directly with Raf, a serine/ threonine kinase, and this induces a transient membrane-anchoring signal. The active Raf kinase binds and activates the dual specificity kin ase, MEK 1 / 2, and this kinase activates ERK 1 / 2 in turn, via phosphorylation of Thr183 and Tyr185. Numerous downstream targets of ERK have been identified, including the 90-kDa ribosomal S6 kinase ($p90^{rsk1}$) and transcription factors such as Elk-1. By analogy with Ras that mediates ERK pathways, two members from another family of small GTP-binding proteins (*R*as *ho*mology, or Rho), called Rac1 and Cdc42, precipitate SAPK signalling. The Rho protein phosphorylates MAP kinase kinase kinase (MAP3K), also referred to as MEKK (p38 cascade) and PAK or MEKK1 (JNK cascade), which phosphorylates the dual-specificity kinases MKK3 or MKK6 (p38 cascade) and a JNK kinase (JNKK) MKK4 or MKK7 (JNK cascade). This dual specificity kinase sometimes is referred to as SEK. Then, in turn, these proteins phosphorylate (activate) JNK or p38 at threonine and tyrosine residues, causing them to translocate to the nucleus where they activate a range of transcription factors, including ATF- 2.

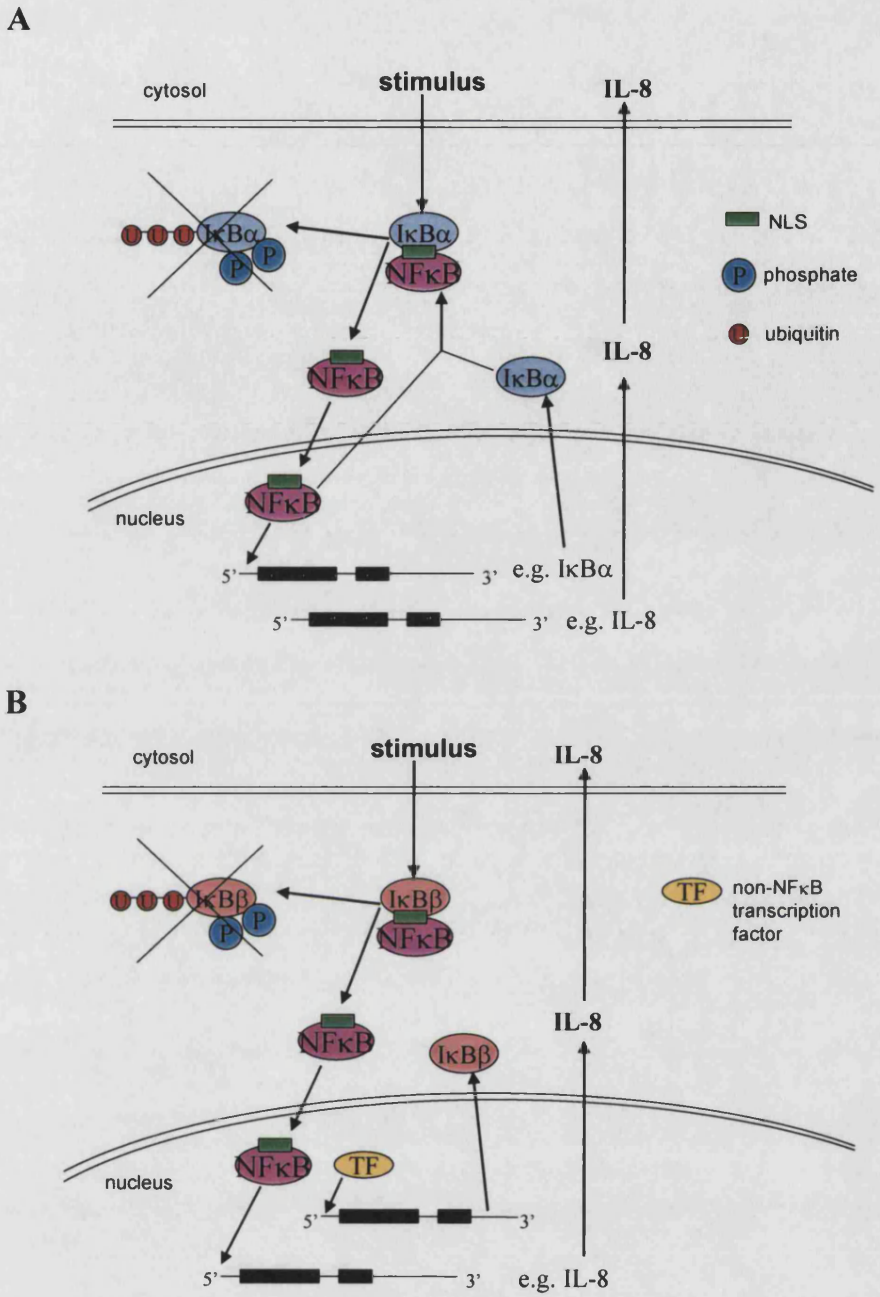


Figure 1.7 Activation of NFκB-mediated gene transcription. In resting cells, NFκB is localised in the cytosol by virtue of its binding the inhibitory protein IκB α, but stimulation of the cell with any of a number of different agonists, such as IL -1β, precipitates phosphorylation of IκBα **(A)** or IκBβ **(B)** and their subsequent degradation via the ubiquitin -26S proteasome pathway. Following its dissociation from IκBα/β, NFκB exposes a nuclear localisation sequence (NLS) and translocates into the nucleus, where it initiates transcription of genes of a number of inflammatory proteins. Resynthesised IκBα masks the NLS once more and terminates transcription, but resynthesised IκB β does not have this capacity. See text for further details.

1.3 The Microbiology of CF

The most striking feature of CF lung infections is that, despite the inactivation of a number of immune responses in the lung, only a narrow spectrum of highly adapted microorganisms repeatedly exploits the immunocompromised pulmonary environment *(160)*. RSV is a common cause of mild lung disease in early childhood of CF and non-CF individuals and coupled with additional factors in the CF lung, pulmonary inflammation caused by RSV may encourage subsequent colonisation by bacterial pathogens *(55;120)*. Initially, *S. aureus* and then *H. influenzae* infect the lungs of very young CF children and these bacteria may continue to cause acute exacerbations throughout the disease course *(160;161)*. Ironically, maintenance antibiotic treatment against these bacteria may help to select for the more troublesome organism *P. aeruginosa*, which is resistant to many antimicrobial agents and is acknowledged to be the most important pathogen in CF respiratory infections *(7;160)*. In the early 1980s, *B. cepacia* emerged as an additional and significant risk to the health of CF patients *(162;163)*, causing much concern amongst patients, carers and microbiologists alike and serving as a warning that this brief list of pathogens is expanding. In recent years, the rate of isolation of additional bacteria, fungi and viruses from CF sputa has increased and *B. cepacia* provides a paradigm of the newly emerging microbiological threats to the CF community. Each of the four major bacterial CF pathogens will be described, followed by a brief discussion of some of the less common and emerging pathogens that constitute the microbiology of CF.

1.3.1 *Staphylococcus aureus*

This Gram-positive, non-motile coccoid human commensal is carried in the upper respiratory tract and nasal passages of as many as 30 % of healthy individuals yet lower respiratory tract infections are rare in the immunocompetent host *(164)*. In contrast, rates of *S. aureus* lower respiratory tract infection are as high as 36 % in the CF community *(165;166)*. In the post-antibiotic era, *S. aureus* no longer is the major cause of mortality in CF patients it once was, although the current crisis with regard to strains such as methicillin-resistant *S. aureus* (MRSA), which survives treatment with most antibiotics, may reverse this trend. *S. aureus* is the first bacterial pathogen to colonise the CF airways and early damage caused to respiratory tissue by this bacterium may encourage subsequent colonisation by *P. aeruginosa (167)*.

1.3.2 *Haemophilus influenzae*

The role of this non-motile Gram-negative bacillus in CF is controversial but probably underestimated, since *H. influenzae* is a normal commensal of the healthy upper respiratory tract *(161;168)*. Following *S. aureus* infection, low numbers of non-capsulated *H. influenzae* may colonise the CF airways but this often remains untreated. Convincing evidence has been published that certain strains of *H. influenzae* elicit proinflammatory chemokines from the respiratory epithelium *(169)*. In addition, *H. influenzae* LPS may damage the pulmonary epithelium and interfere with ciliary beat frequency *(170)*. This latter strategy to subvert host immunity is also employed by *P. aeruginosa (63)*.

1.3.3 *Pseudomonas aeruginosa*

Without question, *P. aeruginosa* is the most significant and troublesome pathogen to infect the CF lung and it is estimated that in some CF clinics *P. aeruginosa* colonisation rates are as high as 80-90 % *(168;171)*. A recent report stated that *P. aeruginosa* infects approximately 21 % of CF infants within 1 year of birth *(171)*. This unusually high prevalence results from an impressive arsenal of virulence determinants secreted by *P. aeruginosa* combined with the ability of this bacterial species to adapt to the hostile environment of the CF lung. This ubiquitous Gram-negative motile bacillus is a common environmental organism *(172)*, which also causes significant opportunistic infections in immunocompromised patients both systemically and locally at most sites of the body, in particular to the corneal epithelium and burn wounds *(173-175)*. *P. aeruginosa* is inherently resistant to many classes of antimicrobial agents and it is a common cause of nosocomial infection by virtue of its ability to survive on surfaces and medical instruments, even after treatment with some disinfectants.

There are three broad strategies by which bacteria may resist the effects of an antibacterial agent namely preventing the drug from reaching its target; modification of the target; and inactivation of the drug. All of these mechanisms have been reported in *P. aeruginosa*. For example, *P. aeruginosa* has a relatively impermeable outer membrane because of its comparative lack of porins as well as the small size of these membrane channels *(176)*. In addition, *P. aeruginosa* expresses at least four tripartite efflux pumps in its outer membrane, which remove from the bacterial cell a wide range of structurally unrelated antibiotics and biocides *(177)*. Furthermore, there is evidence that the

outer membrane pumps act in synergy both with each other and with the inherently low permeability of the *P. aeruginosa* outer membrane *(178;179)*. Other CF pathogens such as *B. cepacia* and *Stenotrophomonas maltophilia* posses homologues of the *P. aeruginosa* efflux pumps *(180)*, providing further evidence of an important role for these molecules in biocide resistance in CF and indicating potential targets for novel drug design. Alterations to DNA gyrase owing to mutations in its gene *gyrA* confer ciprofloxacin resistance on *P. aeruginosa (181)*, whilst target modification also reduces the activity in *P. aeruginosa* of antimicrobial agents such as aminoglycosides.

Inactivation or destruction of some antibiotics by bacterial enzymes is particularly problematic and is exemplified by the production by *P. aeruginosa* of β-lactamases against penicillin and its derivatives *(176;182)*. Other inhibitors of cell-wall synthesis include carbapenems, which are stable to most β-lactamases, although the prevalence of acquired metallo-carbapenemases such as IMP-1 and VIM in *P. aeruginosa* is increasing *(183)*. Furthermore, chromosomal carbapenemases have been identified in *S. maltophilia (183)*, an opportunistic pathogen that poses a potential risk to CF patients. Another example of drug modification by *P. aeruginosa* is the chloramphenicol acetyl transferase (CAT) that adds an acetyl group to, and inactivates chloramphenicol *(184)*. Genes encoding antibiotic resistance are commonly found clustered onto mobile genetic elements such as plasmids and transposons, which permit horizontal transfer of this information amongst different bacterial species. The alarming rate at which these genes may disseminate has prompted the use of combination therapy to reduce the spread of antibiotic resistance, such as an

inhaled aminoglycoside and an oral penicillin *(185)*. *P. aeruginosa* is able to withstand treatment with high doses of antibiotics, and this ability constitutes only one of the multiple and complex mechanisms by which *P. aeruginosa* is able to exploit the CF lung.

1.3.4 The pathogenesis of *P. aeruginosa.*

After gaining entry to the host, flagella propel *P. aeruginosa* through the respiratory tract where this bacterium induces intermittent episodes of pulmonary exacerbation followed by chronic asymptomatic carriage in the lung *(160;186;187)*. *P. aeruginosa* adheres to the lung via several host factors, such as CFTR *(45)*, ganglioside moieties *(67;188)* and mucins *(189)* mediated by bacterial factors such as LPS *(45)*, type IV pili *(188)* and the flagellar cap protein, FliD *(190)*. Terminal sialylation is altered in the CF lung because of impaired pH-dependent sialyltransferase activity in intracellular glycosylation compartments *(67)* so there is an increased expression of asialo-GM1, to which *P. aeruginosa* and other CF pathogens bind with high affinity *(188)*.

Hypersecretion of tracheobronchial mucin in CF *(59;60)*, which is increased further by *P. aeruginosa* binding to the lung epithelium and by certain pseudomonal exoproducts *(64;191;192)*, enhances *P. aeruginosa* colonisation of the lung either because of specific molecular adhesive interactions or simply because the mucin is so sticky. It has been proposed that *P. aeruginosa* binds CFTR and becomes internalised into respiratory epithelial cells and that subsequent epithelial desquamation serves as a bacterial clearance mechanism from the lung, highlighting one way in which the relative absence of CFTR expression on the CF lung epithelium may contribute to the persistence of this

bacterial pathogen *(45;66)*. This view is based on the observations that pulmonary epithelial tissue from CF patients bound and internalised much less *P. aeruginosa* than did non-CF epithelial cells *(193)* and that a synthetic peptide corresponding to the first extracellular loop of CFTR reduced *P. aeruginosa* internalisation by non-CF epithelial cells *(45)*. However, this interpretation remains controversial *(65;68)*.

Virulence factors such as proteases, lipases and haemolysins like rhamnolipid are important during the initial states of *P. aeruginosa* infection *(7;160;194-196)* and these exoproducts act synergistically to induce symptoms of acute infection. Elastase is a potent digestive metalloprotease that is secreted both by *P. aeruginosa* and by frustrated phagocytes in the CF lung and its substrates include complement components, immunoglobulin and elastin *(197)*. Elastase also cleaves host transferrin and lactoferrin to release sequestered iron into the lung *(198)*, where it is scavenged by *P. aeruginosa* pyocyanin, a siderophore that can also immobilise the mucociliary escalator *(63)*. Pyochelin is another *P. aeruginosa* siderophore, which forms complexes with molecular iron called ferripyochelin and, together with pyocyanin, catalyses the production of superoxide and hydroxyl radicals thus causing significant free radical damage to lung tissue *(199;200)*. *P. aeruginosa* produces other siderophores, including pyoverdin and inositol polyphosphates *(201;202)*, which are also important to the pathogen during chronic infection, when the CF lung becomes particularly iron-deplete. Siderophores propagate the respiratory immune response for example, pyocyanin induces epithelial IL-8 production *(203)*, which recruits and activates pulmonary neutrophils.

Exotoxin A is a potent *P. aeruginosa* ADP-ribosylating toxin, which is the product of the *toxA* gene *(204)* and it interrupts protein synthesis via ADP-ribosylation-mediated inhibition of elongation factor-2 (EF-2) *(205)*. Exotoxin A also kills macrophages *(206)* and is important during initial colonisation but shows maximal expression during iron-limitation *(160;207)*.

After repeated episodes of acute pulmonary exacerbation, *P. aeruginosa* becomes established chronically in the CF respiratory tract in an attenuated form. Flagella no longer are expressed *(187;208)*, toxin production is reduced *(160)* and the *P. aeruginosa* LPS loses its O-side chains, assuming a 'rough' phenotype *(209)*. Although the latter change renders *P. aeruginosa* sensitive to complement-mediated killing, the relative inactivity of complement in the CF lung negates this issue *(210)*. Thus, initial chronic pseudomonal colonisation in CF is asymptomatic, although the same organism would produce significant clinical manifestations in an immunocompromised non-CF individual.

Attenuation of virulence correlates with the spontaneous mutation *in vivo* of *P. aeruginosa* into a mucoid phenotype that shows enhanced antibiotic resistance because of a profusely secreted polyanionic exopolysaccharide called alginate and the formation of adherent bacterial microcolonies or 'biofilms' *(211)*, after which eradication of *P. aeruginosa* from the lung is seldom achieved. Alginate is anti-phagocytic and reduces neutrophil chemotaxis despite the potent induction of epithelial IL-8 by this exopolysaccharide *(212)*. Gradually, iron-depletion from the CF lung becomes more significant until it reactivates virulence factor production by *P. aeruginosa.* The host produces high titres of antibodies against a wide range of these virulence determinants,

such as alginate, LPS, iron-regulated membrane proteins and elastase *(160;213)*, an event which correlates with progressive deterioration of pulmonary function and is associated with a poor clinical prognosis that may be caused by excessive immune-complex deposition *(160;214)*.

Alginate allows community growth of a heterogeneous population of *P. aeruginosa* in adherent microcolonies, which develop into mature biofilms, several hundred micrometres deep, as shown in **Figure 1.8**. Water channels bring in nutrients and remove waste products and a steep oxygen gradient exists across the biofilm *(215)*, determining the bacterial growth rate at different locations within the structure and therefore dictating in part the susceptibility to antibiotics of the bacterial cells within the biofilm *(216)*. Biofilm resistance to biocides may also be attributed to a reduced penetration of the drugs into the mucoid matrix because of the impermeable nature of the structure as well as the production of inactivating enzymes by bacterial cells around the biofilm periphery *(216)*. Biofilm growth is concurrent with a stress response that is mediated by the alternative bacterial sigma factors AlgU and RpoS, and is switched on by nutrient limitation and environmental cues via bacterial signal transduction *(217)*. Alginate production, biofilm development and the secretion of extracellular toxins are all highly energy demanding processes so they are regulated tightly by specific bacterial signalling cascades *(218-220)*.

1.3.5 Signalling in *P. aeruginosa*.

Effective attacks on host cells require the contribution of a critical number – or quorum – of bacteria. Gram-negative bacteria secrete small (300-700 Da) molecules called autoinducers (AI) and together with specific AI 'receptors'

(effector molecules), allow neighbouring bacteria to sense and communicate with the surrounding population and enable a co-ordinated attack on the host, once an appropriate density is attained. This signalling mechanism is therefore known as "quorum sensing" (QS) *(221)*.

Chemically, AI molecules have been identified as acyl homoserine lactones (acyl-HSLs) and have no known function other than signalling and both acyl side chain length and substitutions provide specificity for the signal *(215)*. Each acyl-HSL is synthesised by a specific enzyme and detected by its cognate effector molecule. Quorum sensing was first discovered in *Vibrio fischeri* more than 30 years ago *(222)*, in which an acyl-HSL synthase, LuxI, catalyses the synthesis of the AI molecule; and the AI effector, LuxR, serves as a transcriptional activator of density dependent genes *(218)*. LuxR family members possess an N-terminal acyl-HSL-binding domain and a C-terminal DNA binding domain *(215)*. Homologues of these gene products have been found in many Gram-negative species including CepR and CepI in *B. cepacia (223)*, as well as two different QS systems in *P. aeruginosa*, namely the LasI/R and RhlI/R systems *(224)*. RhlI was so-named because it regulates the *rhl* operon that controls rhamnolipid biosynthesis and this enzyme generates mainly butyryl HSL (BHL or C4-HSL) *(215)*. In contrast, LasI synthesises mainly N-(3-oxododecanoyl)-HSL (OdDHL, or 3OC12-HSL) and derives its name from its reported regulation of the *las* operon, which mediates elastase biosynthesis *(215;224)*.

It is now known that these QS systems regulate the expression of many more bacterial genes besides, indeed it has been estimated that as many as 4% of the

approximately 6,000 *P. aeruginosa* genes are under the control of QS *(220)* and there is a strict requirement for the QS systems in virulence. In a murine model of pulmonary infection, LasI⁻ mutants of *P. aeruginosa* were able to invade and survive in lung tissue but did not cause disease *(225)*. Neither did QS mutants of *P. aeruginosa* cause infection in a burned mouse model *(175)*. Co-administration of OdDHL restored the virulence potential of *P. aeruginosa* LasI⁻ mutants *(175)*. Similarly in another study, LasI⁻ mutants resulted in *P. aeruginosa* microcolonies that were unable to develop into a mature biofilm, but addition of OdDHL to the microcolony corrected this defect *(226)*. Resistance to the respiratory burst is also regulated in part by QS *(219)*.

An intriguing study by Singh et al. *(227)* showed that the ratio of BHL to OdDHL determined whether bacterial growth would tend to be planktonic or assume a biofilm formation and this observation could have implications for the treatment of *Pseudomonas* respiratory infection since in general, antibiotics are more effective against planktonic than against biofilm bacterial cells *(216)*. This study was also critically important because it provided the first evidence, in terms of autoinducer ratios, that *P. aeruginosa* grows as a biofilm in the CF lung *(227)*. In addition to the effects of acyl-HSLs on bacterial virulence products, some of these bacterial signalling molecules have direct immunomodulatory effects in the host. For example, there have been several reports that autoinducers stimulate the production of cytokines such as TNF-α and IL-8 *(228;229)*, both of which play an important role in the host immune response to *P. aeruginosa*. Telford's report also indicated that *P. aeruginosa* HSL molecules may affect lymphocyte proliferation *(229)*.

Of particular relevance to CF was the observation that *P. aeruginosa* acyl-HSLs antagonised the expression of P2Y receptors in CF tracheal gland cells. ATP and UTP bind P2Y receptors and activate Ca^{2+}-medaited Cl^- -channels in respiratory cells, thus bypassing the ion channel defect of CFTR *(230)*. Therefore, QS molecules might contribute to the physiological dysfunction within the lung. Exciting possibilities exist for novel antimicrobial therapy directed against bacterial signalling cascades and these approaches have begun to be investigated *(231;232)*. Another sophisticated strategy for evading the host immune response concerns the secretion of a subset of 'stealth' bacterial toxins by *P. aeruginosa* and other Gram-negative bacteria via type III secretion systems.

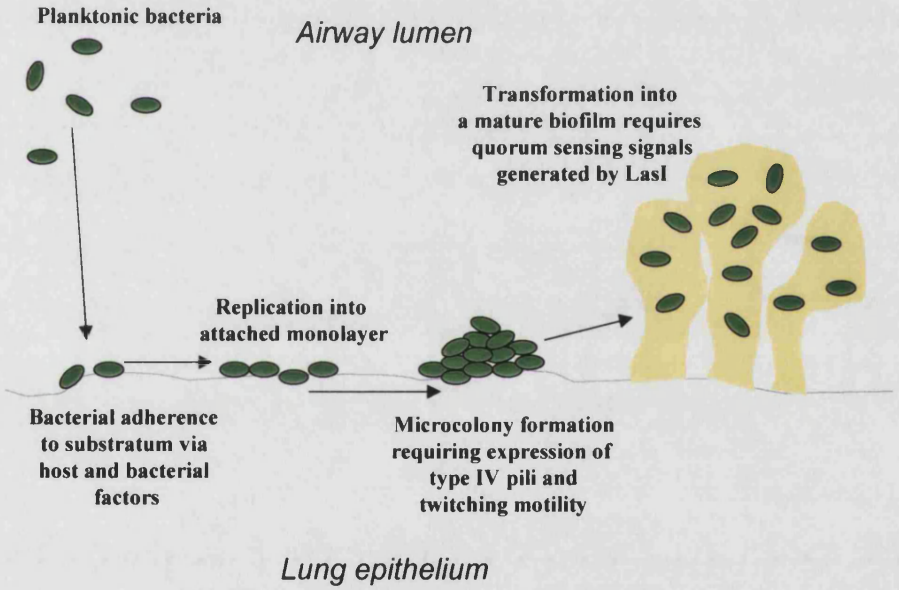


Figure 1.8 Biofilm formation in the lung. Planktonic bacterial cells in the airway lumen attach to the pulmonary epithelium, whereupon they multiply into a monolayer. Bacterial cells express type IV pili, which are responsible for the 'twitching motility' that allows the development of adherent microcolonies *(233)*. Maturation of *P. aeruginosa* microcolonies into tower and mushroom-shaped biofilm structures is dependent upon LasI to generate QS signals.

1.3.6 Protein secretion in Gram-negative bacteria.

Gram-negative bacteria export a diverse range of proteins but utilise only four main pathways *(234;235)*. Type II and type IV secretion mechanisms are referred to collectively as the General Secretory Pathway (GSP) and they employ the *sec*-system. Approximately 30 residues form a signal sequence in the amino-terminal of the protein to be secreted by the GSP. This sequence directs the protein to the periplasmic space, where the amino acid signal is cleaved by peptidases. Finally, the protein is transferred across the outer cell membrane into the extracellular space. Type II secretion is the major pathway by which Gram-negative organisms secrete extracellular degradative enzymes; for example, *P. aeruginosa* exports phospholipase C (PLC), elastase and ETA via this route *(234)*.

In contrast, type I and type III mechanisms are *sec*-independent, they by-pass the periplasm and they do not require the proteins to be tagged with a cleavable amino-terminal signal sequence. The type I pathway mediates secretion of *P. aeruginosa* proteases *(234;235)* and proteins are targeted for type I secretion by a highly specific 60-amino acid export signal that lies in the carboxy-terminal region *(234;235)*. However, the most important way by which pathogenic Gram-negative bacteria export protein is the type III secretion system, which is dedicated to the export of toxic products *(234)*.

Type III secretion was described first in *Yersinia* spp. *(236)*, although this protein export mechanism has been described in a number of other important human, animal and plants pathogens including *Escherichia* spp., *Salmonella* spp., *Shigella* spp. and *Pseudomonas* spp. *(234)*. The secretion apparatus is

highly conserved amongst bacterial species, but heterogeneity amongst secreted proteins creates diverse and distinct infections *(234;237)*. Type III secretion requires cellular contact between the bacterium and its target eukaryotic host cell, resulting in the transcription and translation into protein of approximately twenty clustered bacterial genes, which encode the secretory apparatus, the energy generating system and chaperones, as well as the effector toxins *(234;238)*. This precise cell-to-cell association allows the bacterial proteins to be 'injected' directly into the eukaryotic cytoplasm, circumventing the immune system and making the type III secretion system a highly effective virulence determinant. However, a critically important discovery was made recently, which showed that neutralising antibodies against PcrV created a significant reduction in the toxic effects of exoenzyme S (exoS) *(239)*. PcrV is a *P. aeruginosa* homologue of LcrV in *Yersinia*, which determines the size of the protein translocation channel *(240)* so that this finding may provide a useful strategy for immobilising type III secreted toxins.

Proteins exported by the type III secretion mechanism in *P. aeruginosa* include exoS, exoT, and exoU *(241;242)*, which are toxic by virtue of their remarkable analogy with host signal transduction components and these proteins can interfere with DNA synthesis, host cell viability, cytoskeletal structure and cell matrix adherence *(243;244)*. Based on the genotype of the *exoS* locus, a recent report *(245)* distinguished invasive strains of *P. aeruginosa* (such as PAK and PAO1), which produced exoS but not exoU, from acutely cytotoxic strains (such as PA103), which expressed exoU but not exoS, although long-term exposure to exoS causes increased tissue damage and bacterial dissemination

(246). Both invasive and cytotoxic *P. aeruginosa* phenotypes produce exoT *(245)*.

The *exoS* locus contains several open reading frames (ORFs) and is under the transcriptional control of the global regulator ExsA, encoded by *exsA (237)*, which is required for expression of exoS, exoU and exoT. ExoU is approximately 70-kDa and induces acute cytotoxicity and epithelial damage *(242;245)*, although it is absent from non-cytotoxic (invasive) isolates of *P. aeruginosa (242)*. ExoS is a 49-kDa, biglutamic acid ADP-ribosylating toxin *(247)* that is found in approximately 90 % of clinical isolates *(245)*, an observation that supports the importance of this toxin in *P. aeruginosa* pathogenesis. ExoT is a 53-kDa protein that co-immunoprecipitates with exoS and these proteins share approximately 75 % amino acid sequence homology *(241)*. However, exoT displays only 0.2% of the ADP-ribosylating activity of exoS *(241)*. Mammalian G-proteins (GTPases) are common targets of type III secreted toxins. ADP-ribosylation of the GTPase Ras by exoS interferes with its guanine-nucleotide exchange factor and locks Ras into a state of permanent inactivation *(248)* so that a multitude of host cell signalling cascades and physiological processes may be disrupted *(243;249)*.

In addition to the cytotoxic ADP-ribosyltransferase activity in exoS C-terminal residues, its amino terminal portion is required for export and the aggregation properties of this toxin *(246)* and induces actin rearrangements via Rho GTPase activation *(250;251)*, conferring resistance to phagocytosis. The growing epithelium shows increased sensitivity to exoS, since cell contact and epithelial differentiation appear to protect against the toxicity of this protein *(252)*. This

discovery suggests that exoS would be especially damaging to the regenerating epithelium following tissue injury in CF, but epithelial susceptibility to exoS is not related to the functional expression of CFTR *(252)*. Virulence determinants such as the type III secretion system are encoded by mobile genetic elements called 'pathogenicity islands' *(253)*. These gene clusters can move horizontally between species so that evolutionarily distinct organisms may share significant homology with respect to virulence determinants. Type III secretion has been described in *P. aeruginosa (246)* and *Burkholderia pseudomallei (254)*, but to date this system has not been found in *B. cepacia*. However, there is a strong possibility that *B. cepacia* type III secretion systems will be discovered in the near future.

1.3.7 *Burkholderia cepacia*.

B. cepacia is a Gram-negative bacillus, which is highly motile by virtue of multiple peritrichious pili *(255)*. *B. cepacia* has a relatively plastic genome consisting of up to three circular replicons, which total approximately 7-Mb of DNA *(256)*. Its unusually large genome allows *B. cepacia* to be highly adaptable and exploit unfavourable environments, such as the CF lung. *B. cepacia* is not a common cause of human infection, having been described originally as a phytopathogen causing soft rot in onion bulbs *(257)* and environmental reservoirs of this organism include soil, water, plants and free-living amoebae *(7;160;258)*. However, both CF patients and immunocompromised individuals, such as those with in-dwelling catheters *(259)* and CGD patients *(260)* may suffer from repeated respiratory or systemic infections with *B. cepacia*. Whilst *B. cepacia* colonisation rates are much lower than those of *P. aeruginosa*, reaching 40 % prevalence at most compared with

more than 85 % prevalence of *P. aeruginosa (7)*, three salient features of *B. cepacia* infection account for the increasing anxiety that this bacterial species has caused within the CF community within the last two decades.

1.3.7.1 Certain strains of *B. cepacia* are highly transmissible.

Strain J2315, a genomovar III isolate that is often referred to as the Edinburgh-Toronto (ET) lineage *(261)*, was implicated in a number of epidemic clusters across Europe and the North American continent *(262;263)*. For example, one patient harboured two different strains of *B. cepacia*, but passed only the epidemic strain to his girlfriend *(7)*. Enhanced person-to-person transmission of this epidemic strain is most likely consequent to expression of the 'cable pilus', which binds specifically to cytokeratin 13 on the host respiratory epithelium *(264)*; and non-specifically to mucin *(265)*. Unlike conventional bacterial pili, the cable pilus expresses adhesin proteins along its entire length, augmenting interaction with the uncharacteristically thick mucus blanket in the CF lung. In addition, adjacent bacterial cells could aggregate via cable pili interactions to form protective microcolonies. Globotriosylceramide (Gb_3) is a good candidate for adhesion to host cells of *B. cepacia* strains that do not express the cable pilus strongly *(266)*. Like *P. aeruginosa, B. cepacia* adheres to the lung via asialo-GM1, but lack of competitive binding between these two pathogens implies that one or both of these bacteria use additional receptors on host cells. Moreover, synergistic binding of *B. cepacia* to the host epithelium following prior colonisation with *P. aeruginosa* has been reported *(267)*.

For many years, summer camps performed a vital role within the CF community but the fear of widespread *B. cepacia* epidemics led to the decision in 1993 to

ban many of these important social events *(268)*. Risk factors for the acquisition of *B. cepacia* infection include social contact with an infected individual (particularly a sibling), for example sharing drinking vessels, kissing or more intimate interactions; and recent hospitalisation possibly because of contaminated surfaces or respiratory equipment *(7)*. It has been reported that *B. cepacia* may survive on environmental surfaces for as long as a week *(261)*. In addition, the use of aminoglycosides, underlying pulmonary disease and increasing age are all factors that predispose CF patients to colonisation with *B. cepacia (7)*. Furthermore, patients already colonised with *B. cepacia* are not immune from acquiring additional epidemic strains *(269)*. This discovery highlights the urgent need to develop improved discriminatory typing methods that allow the pathogenic potential of *B. cepacia* to be assessed so that more appropriate segregation policies can be adopted.

Isolated cases of *B. cepacia* infection in the immunocompetent host have also been described, including a non-CF mother who developed *B. cepacia* pneumonia with a genomovar III cable-piliated strain, which shared the same pulsed field gel electrophoresis (PFGE) pattern as a strain cultured from each of her two CF children *(270)*. This latter observation led to the proposal that CF patients colonised with the epidemic strain should be segregated further from all other *B. cepacia*-infected individuals *(269)*. Whilst such measures may seem draconian, there is good evidence that segregation has been successful in reducing the prevalence of *B. cepacia* fatalities *(271;272)*.

Extensive efforts have been made to discriminate between virulent and less pathogenic strains of *B. cepacia*, to permit a more selective segregation strategy,

but with only limited success. To compound the problem, patients may respond differently after colonisation with an identical *B. cepacia* strain. For example, although a number of patients have died within 6-12 months of becoming infected with the most virulent strain, J2315, the index case survived for many years (J.R.W. Govan, personal communication). This important caveat highlights the fact that host factors are as important as bacterial characteristics in determining the clinical progression of an infection in CF *(16)*.

1.3.7.2 Variable pulmonary disease with *B. cepacia.*

The second feature of *B. cepacia* infection that distinguishes it from *P. aeruginosa* concerns the severity of pulmonary disease. Three possible clinical outcomes are observed following *B. cepacia* acquisition: chronic asymptomatic carriage, progressive deterioration over a protracted period, or rapid fatal decline associated with necrotising pneumonia and septicaemia, a condition known as 'cepacia syndrome' *(163)*. Life expectancy may be reduced to just a few weeks after infection with a particularly virulent isolate *(7)*. However, in contrast to *P. aeruginosa* infection, the *B. cepacia* factors responsible for the severe clinical sequelae remain poorly characterised but possible candidates include LPS *(62;273;274)*, a protease that is cross-reactive with antibodies to *P. aeruginosa* elastase *(275)*, lipase *(276)*, exopolysaccharide *(277)* and siderophores *(278)*.

B. cepacia LPS is more endotoxic than that of a number of other CF pathogens and displays similar toxicity to *E. coli* LPS *(273)*. For example, *B. cepacia* LPS primed and elicited more TNF-α from monocytes than LPS from either *P. aeruginosa* or *S. maltophilia (62;273;274)*. In addition, a specific humoral

immune response is mounted against *B. cepacia* LPS that does not cross-react with *P. aeruginosa (279)*. Taken together, these observations suggest a potentially important role for LPS in the onset of 'cepacia syndrome' that might be mediated by immune complex deposition in the lung and exacerbated by TNF-α. In addition to endotoxin, approximately 40 % of *B. cepacia* strains secrete proteins with haemolytic activity *(276;280)* and one such protein from the genomovar III strain, J2315, induced neutrophil degranulation at high concentrations and apoptosis of neutrophils at lower (1 μg/ ml) concentrations *(280)*.

Mucoid isolates of *B. cepacia* are much less common than mucoid *P. aeruginosa* and the composition of the respective exopolysaccharides differs greatly. *P. aeruginosa* alginate consists of repeating units of β-$_D$-mannuronic and α-$_L$-guluronic acids *(7)*, whilst *B. cepacia* exopolysaccharide contains rhamnose, mannose, galactose, glucose and glucuronic acid *(281)*. Furthermore, hyperosmolarity and nitrogen limitation stimulate alginate production by *P. aeruginosa*, but these conditions do not induce *B. cepacia* exopolysaccharide production *(7)*. Convincing data have been presented for a pathogenic role of *P. aeruginosa* exopolysaccharide, as described previously, but the evidence for *B. cepacia* exopolysaccharide as a virulence determinant remains circumstantial. However, a role for *B. cepacia* siderophores in CF lung disease has been demonstrated.

Four major siderophores have been identified in *B. cepacia*, namely ornibactin, salicylic acid, pyochelin and cepabactin *(282-285)*. Cepabactin appears not to be produced by clinical isolates from CF patients *(285)*, whilst strong evidence

for the virulence potential of pyochelin has been presented. Exogenous pyochelin augmented the virulence potential of pyochelin-negative isolates of *B. cepacia (286)*, which were associated with less severe pulmonary disease *(284)*. Moreover, patterns of siderophore production correlated well with typing fingerprints of clinical isolates, so that this family of pathogenic factors may help to determine the virulence of particular *B. cepacia* strains *(278)*.

1.3.7.3 Multiple drug-resistance of *B. cepacia*.

The final alarming property of *B. cepacia* is its intrinsic resistance to many antibiotics, including several potent anti-pseudomonal drugs *(185)*. This feature may be attributed in part to its impermeable outer membrane *(287;288)*, which has been reported to be ten-fold less permeable than that of *E. coli (289)*; and also to its altered LPS structure *(288)*. *B. cepacia* possesses an antibiotic efflux pump analogous to the multi-drug resistance pumps of *P. aeruginosa* conferring resistance to trimethoprim, ciprofloxacin and a number of penicillins *(290)*. In addition, Hancock identified a *penA* gene that is induced in *B. cepacia* by β-lactams *(291)* and allows penicillin G to be utilised as a substrate *(292)*. However, *B. cepacia* does not produce chloramphenicol acetyl transferase (CAT) that is found in 50-85 % of *P. aeruginosa* isolates *(184)*. The reduced outer membrane permeability and altered LPS composition also protect *B. cepacia* against endogenous defensins *(102)* whilst *P. aeruginosa* remains sensitive to these natural antibiotics *(69)*.

Biofilm formation by *B. cepacia* under nutrient limiting conditions in the CF lung *(293)* provides *B. cepacia* with an additional anti-bactericidal mechanism and may contribute to poor predictive *in vitro* antibiotic susceptibility testing.

This problem highlights the need for a reproducible and physiologically relevant model to predict the efficacy of antibiotics *in vivo*, a challenge that is beginning to be addressed *(293)*. Conversely, lactoferrin enhanced the antibacterial activity of doxycycline, chloramphenicol and rifampicin against *B. cepacia* and *P. aeruginosa (294;295)*, an important finding in light of the elevated production of lactoferrin in the CF lung to approximately 0.9 g/ L *(294)*.

Intracellular growth in macrophages is an important way in which *B. cepacia* can avoid antibiotic exposure and evade the host immune response *(133)*. The respiratory burst in activated macrophages generates reactive oxygen species such as superoxide ($O_2^{-\cdot}$) radicals and hydroxyl ($OH^{\cdot}$) radicals, which are potent bactericidal agents. However, *B. cepacia* secretes melanin, a brown pigment that scavenges and detoxifies $O_2^{-\cdot}$ radicals *(296)*, as well as producing catalase to detoxify $OH^{\cdot}$ radicals *(101)*, allowing *B. cepacia* to overcome this host defence mechanism *(296)*.

Reports that NO production is diminished in CF *(86)* may begin to explain the affinity of *B. cepacia* for the CF lung, since NO may potentiate superoxide- and hydrogen peroxide-mediated killing of *B. cepacia (84)*. Patients with CGD are unable to mount a phagocytic respiratory burst because they lack the enzyme NADPH reductase, relying instead on non-oxidative immune responses such as defensins. Since this bacterium is resistant to these non-oxidative killing mechanisms, CGD patients also suffer from recurrent infection with *B. cepacia.* Coupled with several observations that *B. cepacia* can invade and replicate within the respiratory epithelium *(297-299)*, which is an immunologically-privileged site, a model is evolving in which *B. cepacia* continually stimulates

an aggressive immune response without itself being killed, causing tissue destruction and impairing pulmonary function within the CF lung. This intracellular growth scenario highlights the potential relevance of attempting to modulate the immune response towards T-cell-mediated mechanisms *(135)*.

1.3.8 The evolving microbiology of CF.

Surprisingly few microorganisms infect the CF lung and it is particularly noteworthy that classical respiratory pathogens such as *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* seldom cause acute exacerbation *(160)*. However, the rapid increase in *B. cepacia* infection in CF patients during recent years has alerted microbiologists and clinicians to the potential threat to the CF community of additional opportunist and multi-drug resistant (MDR) pathogens, which have been isolated with increasing frequency from CF sputa *(168)*. This problem may be related in part to periods of prolonged hospitalisation, since repeated exposure to sub-lethal concentrations of biocides selects for resistant isolates and may encourage horizontal spread of antibiotic resistance genes. Indeed, the incidence of nosocomial infection with MDR pathogens has continued to escalate in recent years and is well documented. In the same way, aggressive antipseudomonal therapy may have accelerated the acquisition and spread of novel MDR organisms in CF patients.

Gram-negative *S. maltophilia* (formerly *Xanthomonas maltophilia*) is a notable example of an MDR nosocomial bacterial opportunist that can pose particular problems in the hospital setting and this organism has been isolated with increasing frequency from the CF lung *(7)*. Although there is no evidence that *S. maltophilia* secretes toxic exoproducts *(273)* or that *S. maltophilia* LPS

displays any inflammatory potential *(300)*, long-term survival following *S. maltophilia* colonisation of CF patients with significant underlying respiratory disease was reduced to just 40 % in one study, compared with 72 % in matched non-colonised patients *(168)*. Furthermore, *S. maltophilia* shows high rates of person-to-person transmission *(273)* and a retrospective study has revealed an inverse correlation between prolonged *S. maltophilia* colonisation and lung function *(301)*.

Other microorganisms that have been isolated intermittently from CF sputa include *Ralstonia pickettii, Bordetella* spp., *Aspergillus* spp. and *Alcaligenes xylosoxidans* and their pathogenic potential has been investigated *(168;300)*. For example, *R. pickettii* LPS increased TNF-α and IL-8 production significantly in whole blood and a macrophage-derived cell line, indicating the inflammatory potential of this organism *(300)*. In light of the relatively rapid and significant emergence of *B. cepacia*, it would seem prudent to monitor these potential pathogens amongst the expanding CF population.

1.4 Aims and Objectives.

The main purpose for this investigation was to measure pro- and anti-inflammatory cytokine production, and Cl^- channel activity of the respiratory epithelium following stimulation with *P. aeruginosa* and *B. cepacia.* By utilising two pairs of airway epithelial cell lines, the objective was to determine whether any of these responses was dependent upon the functional expression of CFTR. In addition, attempts were made to characterise signalling pathways

involved in IL-8 production to identify potential targets for therapeutic intervention to reduce the chronic bronchiectasis associated with lung infection in CF.

CHAPTER 2:

Materials

2.1 Chemicals

All chemicals were purchased from Sigma Chemical Co., unless stated otherwise, and were of Analytical Reagent Grade or equivalent.

2.2 Distilled water

Freshly double distilled water (ddH_2O, referred to as Milli-Q water) was taken from the Milli-Q filtration unit and steam-sterilised in 500 ml volumes. Sterile Milli-Q water was used to prepare solutions for epithelial cell and bacterial cell culture and experiments.

2.3 Liquid N_2

Liquid N_2 was purchased from BOC gases, and N_2 cell-storage tanks were re-filled regularly.

2.4 Solutions

Sterile PBS *(302)* was purchased from Gibco at 10x and diluted aseptically into sterile Milli-Q as required; PBS for non-sterile use was prepared as shown in **Table A1**. Solutions used in multiple procedures are detailed in the appendix, whilst those solutions specific for individual experimental procedures are described in the appropriate sections.

2.5 General disposables

Polypropylene tubes (0.5 ml and 1.5 ml) were purchased from Elkay and automatic pipette tips were obtained from Greiner Labortechnik Ltd. Sterile

Pasteur pipettes were obtained from Alpha Laboratories Ltd. Cryovials were from Nunc.

2.6 Epithelial Cell Culture

2.6.1 Epithelial cells

Details of epithelial cell lines are shown in **Table 2.1**.

2.6.2 Epithelial cell culture reagents

The following cell culture reagents were purchased from Gibco-Life Technologies:

- Dulbecco's Modified Eagle Medium, DMEM *(303)* with 1.0 g/ L D-glucose, 0.11 g/ L sodium pyruvate, 0.58 g/ L L-glutamine and 4.0 mg/ L pyridoxine;
- Minimum Essential Medium, MEM *(304)* with Earle's salts and 0.406 g/ L L-alanine-L-glutamine (Glutamax-I);
- MEM non-essential amino acids (100x – **Table A2**) *(304)*;
- Foetal bovine serum (heat inactivated);
- Penicillin (10 000 U)/ streptomycin (10 mg) solution;
- Fungizone (amphotericin B, 250 μg/ ml);
- Trypsin (0.5 g/ L)/ EDTA (0.2 g/ L) solution in Modified Puck's Saline-A
- MEM D-Val Medium *(304)* with Earle's salts, 0.292 g/ L L-glutamine and 92.0 mg/ L D-valine (substituted for L-valine);

- Dulbecco's phosphate buffered saline (PBS) and PBS without calcium and magnesium (PBS-A) *(302)*, (both 10x solution);
- HEPES (1 M) solution

Hygromycin B (400 mg/ ml) was purchased from Calbiochem-Novabiochem and Vitrogen 100 (bovine dermal collagen types I and III, 3.0 mg/ ml) was obtained from Cohesion Technologies. Gentamicin sulphate and protease type XIV both were from Sigma.

2.6.3 Disposable equipment for epithelial cell culture

All disposables for tissue culture had been gamma-irradiated by the manufacturer before purchase, unless stated otherwise. Standard vented 25 cm^2 (T25), T80 and T175 tissue culture flasks were purchased from Nunc. Sterile 150 ml, 100 ml, 60 ml, 30 ml and 7 ml containers were purchased from Bibby Sterilin. Falcon brand 50 ml tubes were from Becton Dickinson. Cell scrapers were from Costar. Non-sterile multi-well strips and frames were purchased from Greiner. Glass coverslips (13 mm diameter, No. 1), also non-sterile, were manufactured by Chance Propper and purchased from Richardson's of Leicester.

2.7 Bacterial Cell Culture

2.7.1 Bacterial strains

Details of bacterial strains used in this study are shown in **Table 2.2.**

2.7.2 Bacterial growth media

Bacterial media were prepared as outlined below, steam sterilised and stored at 4-8°C.

2.7.3 Complex media

Luria Bertani (LB) Broth	LB base (Difco)	25 g
	ddH_2O	to 1 000 ml
Nutrient Agar	Nutrient Agar (Difco)	28 g
	ddH_2O	to 1 000 ml

2.7.4 Chemically defined media (CDM)

Succinate minimal medium (SMM)	K_2HPO_4	6.0 g
(305)	KH_2PO_4	3.0 g
	$(NH_4)_2SO_4$	1.0 g
	$MgSO_4.7H_2O$	0.2 g
	Sodium succinate	4.0 g
	ddH_2O	to 1 000 ml
Casamino Acids (CAA)	Casamino acids (Difco)	0.1 g
	ddH_2O	to 10 ml

(stored in aliquots of 1 ml after sterilisation)

M9 minimal medium

(306)	M9 minimal salts (Sigma)	10 g
	(see Table A3)	
	ddH_2O	to 1 000 ml
	[pH 7.0]	

2.7.5 Disposable equipment for bacterial cell culture

Petri dishes were obtained from Bibby Sterilin and spectrophotometer microcuvettes were from Elkay Products Inc.

2.8 General Equipment

Automatic pipettes: Gilson pipetman P-2, P-10, P-20, P-100, P-200, P-1000, P-5000 (Anachem Ltd.)

Filtration equipment: 25mm Swinnex units 0.2 μm pore size (Millipore). Disposable 0.2 μm pore size filter bottle tops (Nalgene, purchased from BDH/ Merck Ltd.).

Gel electrophoresis apparatus: Bio-Rad Mini Protean II Cell (Bio-Rad Laboratories Ltd.).

Heated water bath: Grant JB2 (Grant Instruments)

Heating block: Techne Dri-Block DB-2A (Techne Cambridge Ltd.)

Incubators: New Brunswick Scientific Controlled Environment Incubator Shaker (New Brunswick Scientific)

Magnetic stirrer: Stuart Magnetic stirrer (Stuart)

pH meter: Corning pH meter 240 (Ciba Corning Diagnostics Ltd.)

Power supply: Bio-Rad 200/ 2.0 power supply (Bio-Rad Laboratories Ltd.)

Water supply: Milli-Q Plus P.F. (Millipore)

Whirlimixer: Fisons Whirlimixer WM/ 250/ SC/ P (Fisons)

Table 2.1. Details of epithelial cell lines and growth media / supplements.

Synonym	Cell Line	References	Description	Growth medium	Supplements
HTE	9HTEo$^-$	*(307)*	Non-CF phenotype; SV40 transformed	MEM with L-alanine-L-glutamine	Present in all cell-line media
CFTE	ΣCFTE29o$^-$	*(308)*	ΔF508 / ΔF508 CF phenotype; SV40 transformed		
16-HBE	16HBE14o$^-$	*(309)*	Non-CF phenotype; SV40 transformed	DMEM with glucose, sodium pyruvate, glutamine and pyridoxine	10 % v/v FCS 1x MEM non-essential amino acids 100 U/ml penicillin 100 µg/ml streptomycin 500 ng/ml amphotericin B
pCEP-2	9HTEo$^-$ /pCEP-2	*(310)*	non-CF phenotype; HTE containing pCEP4β plasmid	DMEM with glucose, sodium pyruvate, glutamine and pyridoxine	
pCEP-RF	9HTEo$^-$ /pCEP-RF	*(310)*	ΔF508 / ΔF508 -like phenotype; HTE containing pCEP4β plasmid	+ 80 µg/ml hygromycin	

Table 2.2. Details of bacterial strains and selective media.

Synonym	Strain	Reference	Description	Selectivity agents added to SMM + CAA
B. cepacia				
JL21	J2315	*(262)*	Highly transmissible, virulent clinical isolate	None
P. aeruginosa				
PAO1	PAO1 (ATCC 15692)	*(311)*	Invasive, non-mucoid laboratory strain	None
PAK 1	PAK	*(312)*	Invasive, non-mucoid laboratory strain	None
PAK 2	PAK*exsA::Ω*	*(312)*	*exsA* mutant of PAK	[a]200 μg/ml St + 200 μg/ml Sp
PAK 3	PAK*exoS::ΩexoT::Gem*	*(312)*	*exoS/exoT* double mutant of PAK	200 μg/ml St + 200 μg/ml Sp + 200 μg/ml Gm
PAK 4	PAK*exoS::ΩexoT::Gem*(pDN19)	*(312)*	*exoS/exoT* double mutant of PAK, containing a vector	[b]100 μg/ml Tet
PAK 5	PAK*exoS::ΩexoT::Gem*(pHW9948)	*(312)*	*exoS/exoT* double mutant of PAK, containing *exoS* clone	100 μg/ml Tet
PAK 6	PAK*exoS::ΩexoT::Gem*(pHW9949)	*(312)*	*exoS/exoT* double mutant of PAK, containing *exoT* clone	100 μg/ml Tet

[a]Key: St – Streptomycin
Sp – Spectinomycin
Gm – Gentamicin
Tet - Tetracycline

[b]Tet was added from concentrated ethanolic stock

CHAPTER 3:

Experimental Methods

3.1 Epithelial Cell Culture.

In all cases, epithelial cell culture media and supplements were purchased from Gibco-Life Technologies and epithelial cells were grown on collagen gels, except when described to the contrary.

3.1.1 Epithelial cell lines.

Two pairs of transformed, human tracheal epithelial cell lines were used routinely in this work. Control cells, 9HTEo$^-$ (HTE) *(307)*, and CF-phenotype cells, ΣCFTE29o$^-$ (CFTE) *(308)*, both were gifts from D.C. Gruenert, University of California, San Francisco, USA. The second pair of cell lines was derived from the HTE cell line by transfection with the pCEP4β plasmid *(310)*. Control cells (pCEP-2) consist of HTE cells transfected with the empty vector; whilst the plasmid in CF transfectants (pCEP-RF) expresses multiple copies of CFTR R-domain, conferring a ΔF508 / ΔF508-like CF phenotype on pCEP-RF cells. pCEP cells were gifts from P.B. Davis, Case Western Reserve University, Ohio, U.S.A. Details of epithelial cell lines are summarised in **Table 2.1.**

3.1.2 Maintenance of cell lines.

Epithelial cells were grown for at least 5 days in standard vented tissue-culture flasks (Nunc) before they were seeded for experiments. CFTE cells were maintained in T175 flasks to generate sufficient cells for each experiment. All other epithelial cells were maintained in T80 flasks. HTE and CFTE cells were grown in MEM *(304)* containing 0.406 g/ L L-alanine-L-glutamine (Glutamax-I); and pCEP cells were grown in DMEM *(303)* containing 1.0 g/ L D-glucose, 0.11 g/ L sodium pyruvate, 0.58 g/ L L-glutamine, 4.0 mg/ L pyridoxine and

80 μg/ ml hygromycin B (Calbiochem) to maintain the plasmid. Both MEM and DMEM were supplemented as shown in **Table 2.1**. Media containing all of these supplements are referred to as "10 % complete" and, unless stated otherwise, 10 % complete medium was used for all epithelial cell procedures. Medium was aspirated from the adherent epithelial cells every 2-3 days and replaced with fresh until the cells were almost confluent. The monolayer was harvested and seeded into dishes or multi-well plates for experiments, and propagated in fresh flasks.

3.1.3 Harvesting epithelial cells.

Epithelial monolayers were washed with 10-15 ml Ca^{2+}- and Mg^{2+}- free PBS (PBS-A) three times and incubated with 2-3 ml trypsin (0.5 μg/ ml) / EDTA (0.2 μg/ ml) at 37°C for 2-3 min until cells began to round up and dislodge. Volumes of PBS-A and trypsin / EDTA were adjusted according to monolayer unit area. Removal of epithelial cells from the flask and separation from each other was completed by dispersion through a Pasteur pipette. Trypsinisation was terminated by addition of 25 ml medium and the epithelial cell suspension was transferred to a 30 ml tube (Bibby Sterilin). Epithelial cells were centrifuged at 300 x **g** for 10 min and the resulting pellet was suspended in 1 ml medium for the cells to be counted. After counting, the epithelial cell suspension was diluted to the required concentration and seeded at the appropriate density into the relevant vessels. Culture supernatant was aspirated every 2-3 days, and replaced with fresh medium.

3.1.4 Epithelial cell viable counts using a haemocytometer.

Approximately 40 µl of epithelial cell suspension was diluted with an equal volume of 0.4% w/v trypan blue in PBS, placed into a haemocytometer counting chamber and overlaid with a glass coverslip. All of the cells in opposing large corner squares of the haemocytometer grid were counted such that at least 100 viable cells had been recorded. Cell density was calculated as follows:

$$\text{cells / ml} = \frac{\text{cells counted x dilution factor x } 10^4}{\text{nr. squares counted}}$$

3.1.5 Cryopreservation of epithelial cells.

Epithelial cells were grown in T175 flasks until confluent. The monolayer was harvested and suspended in 7.5 ml medium. A volume (400 µl) of the suspension, containing $2x10^5$ – $8x10^6$ cells, was transferred into cryovials (Nunc) containing 0.5 ml dimethyl sulphoxide (DMSO, Sigma) and 0.1 ml FCS. Cryovials were placed in a "Cryo 1°C freezing container" (Nalgene) with isopropyl alcohol (BDH) at -80°C overnight, which cools cells at a rate of -1°C per min to prevent cryolysis. Frozen epithelial cells were stored in liquid N_2. One vial of epithelial cells from each batch was thawed the following day to confirm that the freezing process had been successful.

3.1.6 Thawing epithelial cells.

Epithelial cells were removed from the liquid N_2 store and part-thawed quickly at 37°C. Cryovial contents were transferred immediately to 25 ml warmed medium and centrifuged at 300 x g for 10 min. The pellet was resuspended in

10 ml medium and transferred to a coated tissue culture flask for growth to confluence before harvesting and re-seeding for experiments. Medium was changed 24 h after thawing to remove final traces of DMSO from the flasks.

3.1.7 Collagen-gel preparation.

All glassware and plasticware onto which cells would be seeded was coated with a solution of 0.2 mg/ ml collagen (**Table 3.1**). After removal of the excess collagen solution, vessels were left to dry overnight, wrapped aseptically and stored at 4-8 °C.

3.1.8 Preparation of multi-well strips for tissue culture.

Non-sterile multi-well strips and frames were placed in a laminar flow hood and drenched in 70 % v/v ethanol. Once the ethanol had evaporated, the strips and frames were exposed to UV radiation overnight. The following day, the strips were assembled aseptically into the frames, coated with collagen and stored at 4-8 °C.

3.1.9 Preparation of glass coverslips for tissue culture.

Non-sterile glass coverslips were soaked in 70 % v/v ethanol overnight. The following day, coverslips were transferred aseptically, one each into the wells of a 24-well plate in a laminar flow hood. After evaporation of the ethanol, coverslips were coated with collagen then the plates were sealed and stored at 4-8°C.

3.2 Bacterial Cell Culture.

3.2.1 Details of strains.

A virulent and highly transmissible clinical isolate of Burkholderia cepacia, strain J2315, was a generous gift from J.R.W. Govan, University of Edinburgh, UK. Two invasive non-mucoid laboratory strains of *P. aeruginosa* were used in this study: PAO1 (ATCC 15692) *(311)* was obtained from American Type Culture Collection; and PAK and isogenic mutants derived thereof *(312)* were kind gifts from Dr. S. Jin, University of Florida, U.S.A. Mutations had been introduced into PAK genes encoding proteins of the Type III secretion system apparatus *(312)*, and five such mutants were used in this work. Details of bacterial strains and selective media are summarised in **Table 2.2.**

3.2.2 Maintenance of bacterial strains.

3.2.2.1 Cryopreservation of bacterial cultures.

Cultures were grown overnight in Luria Bertani (LB) broth (Difco) containing the appropriate selective antibiotics (**Table 2.2**). A volume of the overnight culture was mixed with sterile glycerol (20% v/v), transferred into cryovials and stored at -80°C.

3.2.2.2 Stock plates.

Strains were cultured from frozen stocks onto nutrient agar with antibiotic selection as required. *P. aeruginosa* plates were stored at 4-8°C and *B. cepacia* plates were stored at room temperature for up to one week, before re-streaking from frozen. Agar plates containing light-sensitive tetracycline were wrapped in foil to store in the dark.

3.2.3 Preparation of Glassware.

All glassware for bacterial culture was soaked overnight in 0.01% w/v EDTA to remove Fe^{3+} and rinsed at least three times in MilliQ water. Glassware was dried and oven-sterilised as usual.

3.2.4 Broth Culture of Bacteria.

All strains were grown in succinate minimal medium (SMM) supplemented with casamino acids (CAA, 0.01 % w/v) [detailed in the previous chapter] at 37°C, and selected as shown in **Table 2.2.** The stock plate was brought to room temperature and used to inoculate pre-warmed culture medium. Inoculated cultures were grown overnight with shaking (200 rpm) in thick-walled baffled glass flasks and those cultures containing tetracycline were covered completely with foil.

3.2.5 Viable counting of stationary phase broth cultures.

Each bacterial strain culture was diluted in M9 salts (**Table A3**) and the OD_{550nm} (optical density at 550 nm) was measured spectrophotometrically (Milton Roy Spectronic 601). Broth cultures were diluted in a 10-fold series into M9 and 100 µl were plated in triplicate onto nutrient agar, for two consecutive dilutions. Plates were incubated overnight (*P. aeruginosa*), or for 48 h (*B. cepacia*), at 37°C and the mean number of colony forming units (cfu) from triplicate plates was noted in each case. OD_{550nm} was calibrated against the corresponding mean cfu so that viable bacterial cell density (cfu/ ml) could be estimated from OD_{550nm} for future cultures of each strain.

3.3 Epithelial - Bacterial Cell Co-incubation.

OD_{550nm} of an overnight bacterial culture in stationary phase was measured. The culture was diluted in M9 and added (10 % v/v) to sub-confluent epithelial cells in multi-well dishes or petri dishes, to give the required multiplicity of infection (moi). Epithelial cell and bacteria were transferred to a small mobile incubator unit (Billups Rothenberg) and gassed for 1-2 min with 5% CO_2: 95 % air. The unit was sealed and placed on a rotating platform at no more than 25 rpm. Co-cultures were incubated at 37°C. If bacterial cells or supernatant alone were to be used to stimulate epithelial cells, 1 ml of the neat whole cell suspension was microfuged at 5,900 x **g** for 10 min. The supernatant (1 ml) was transferred to a 1.5 ml polypropylene tube and diluted accordingly. Pellets of bacteria were suspended gently in 1 ml M9 and diluted as required.

3.4 Ion Channel Studies.

3.4.1 Fluorimetric analysis using PTI fluorimeter. Cells were seeded (1 x 10^5) onto 13 mm diameter glass coverslips and grown for at least 4 days until patches of confluent cells were visible. Cells were loaded with 10 mM N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE, Molecular Probes) in serum-free medium for 4 h at 37°C. The principle of this assay is shown in **Figure 3.1**. As required, each coverslip was washed in Cl^- buffer (**Table 3.2**) three times, assembled into the coverslip perfusion chamber and overlaid with 200 µl Cl^- buffer. All buffers were warmed before use. Cells were brought into view under a x40 oil immersion objective on an inverted Nikon Diaphot 300 microscope attached to a Photon Technology International (PTI) Inc. dual-wavelength fluorimeter. The viewed area was adjusted by way

of moveable slits to reduce background auto-fluorescence and epithelial cell fluorescence was measured using the PTI fluorimeter (365 nm excitation; 465 nm emission) at room temperature, in a darkened room. Each sample was read for 1 min to obtain a stable baseline for comparison, corresponding to unstimulated cells, F_{min}. Cl^--buffer was aspirated and replaced with 100 µl Cl^--free (NO_3^-) buffer (**Table 3.2**) three times, and fluorescence was measured for 1 min to monitor Cl^- leakage from unstimulated cells. One hundred microlitres of agonist were added cell fluorescence was recorded for 5 min, or for 10 min in the case of forskolin, which has longer reaction kinetics. Fifty microlitres of 0.5 mg/ ml digitonin (Sigma) in 70 % ethanol were added at the end of each stimulation to lyse the cells and obtain a maximum fluorescence reading, F_{max}, corresponding to $[Cl^-]_i=0$. Percentage change in fluorescence, ΔF, was calculated as:

$$\Delta F = (F_t - F_{min}) / (F_{max} - F_{min}) \times 100\ \%$$

where F_t is the fluorescence at time, t; and the degree of dye loading was calculated as the difference between F_{min} and F_{max}.

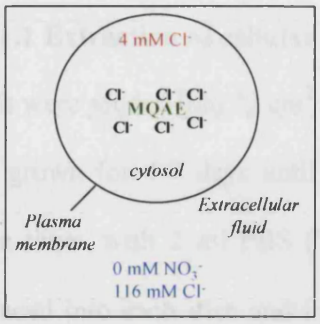
3.4.2 Fluorimetric analysis using Fluoroskan Ascent plate-reader.

Cells were seeded into multi-well strips (1×10^4 / well) and grown for at least 4 days. Once confluent patches of cells were apparent, cells were washed three times with 200 µl PBS and incubated with 10 mM MQAE in serum-free medium for 4 h. Each strip of cells was removed as required, washed three times with 200 µl Cl^- buffer and overlaid with 50 µl Cl^- buffer. Fluorescence

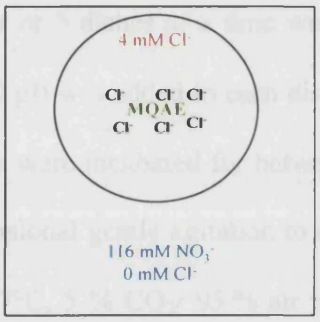
was measured (355 nm excitation, 460 nm emission) using the semi-automated Fluoroskan Ascent 2.2 plate reader with Ascent software Version 2.4, for 2 min. Cells were rinsed three times with 200 µl NO_3^- buffer, overlaid with 50 µl NO_3^- buffer and cell fluorescence was measured for 1 min. One hundred microlitres of agonist were added and fluorescence was monitored for 5 min. Cells were lysed with 100 µl of 0.5 mg/ ml digitonin to obtain F_{max} and the data were analysed to calculate the change in fluorescence over time, as described previously.

3.4.3 Radioisotopic analysis.

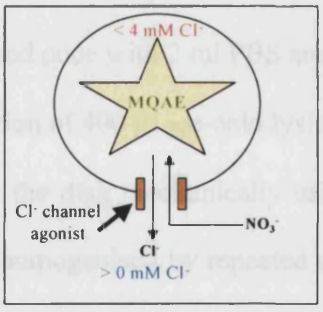
Cells were grown in small petri dishes for at least 4 days, rinsed three times with 2 ml efflux buffer **(Table 3.3)** and incubated at 37°C for 1 h with 5 µCi ^{36}Cl (Amersham) in efflux buffer. Cells were rinsed rapidly with 2 ml efflux buffer three times then 1 ml fresh efflux buffer was added and removed for scintillation counting after 1 min. Twice more, fresh efflux buffer (1 ml) was added for 1 min then removed for counting. One millilitre of fresh efflux buffer containing agonist at the required concentration was added and removed after 1 min for the next 9 min. Finally, cells were rinsed three times with 2 ml ice-cold Mg^{2+}-sucrose wash buffer **(Table 3.4)**. Cells were lysed overnight with 0.1 M NaOH (1 ml) and the lysates were collected for counting. All samples were diluted with 10 ml scintillant (Optiphase Hi-safe), stored in the dark for 2 h and then read using a liquid scintillation counter (LKB Wallac Rack II beta (1207), Wallac), with a darkened lid, for 2 min per sample. Efflux of ^{36}Cl was expressed as a function of time and rates of efflux were compared for different agonists.



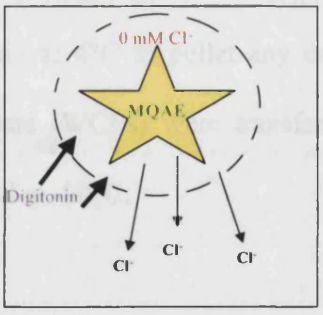
A: MQAE is loaded into the epithelial cell where its fluorescence is quenched by intracellular Cl^-. The difference in Cl^- concentration between the cytosol (~4mM) and extracellular fluid (110-130mM) is maintained by the plasma membrane.



B: Cl^- is removed from the extracellular fluid and replaced with NO_3^-. This situation corresponds to minimum fluorescence.



C: Activation of one or more groups of Cl^- channel in the plasma membrane allows Cl^- to flow out of the cell and NO_3^- to flow into the cell down their respective concentration gradients. As MQAE inhibition by Cl^- decreases so its fluorescence intensity increases. NO_3^- does not quench MQAE fluorescence.



D: Digitonin permeabilises the cell membrane so that the intracellular Cl^- concentration = 0 mM. This situation corresponds to maximum fluorescence.

Figure 3.1 Effects of Cl^- on MQAE fluorescence in cells.

3.5 MAP Kinase Pathway Studies.

3.5.1 Western blotting.

3.5.1.1 Extraction of cellular protein.

Cells were seeded into 22 cm^2 petri dishes (Nunc) at $1x10^5$ / dish or $2x10^5$ / dish and grown for 4-7 days until almost confluent. The monolayer was washed three times with 2 ml PBS (**Table A1**) then 1.8 ml serum-free medium was replaced into each dish and incubated overnight at 37°C, 5 % CO_2/ 95 % air. Four or 5 dishes at a time were removed from the CO_2 incubator and agonist (200 µl) was added to each dish, which were swirled gently to mix. Stimulated cells were incubated for between 30 sec and 15 min at room temperature, with occasional gentle agitation to ensure adequate mixing; or for 30 min or 60 min at 37°C, 5 % CO_2/ 95 % air in a modular incubator chamber, with rotation at 25 rpm. The supernatant was discarded quickly by inversion and cells were washed once with 2 ml PBS and transferred to ice immediately, followed by the addition of 400 µl ice-cold lysis buffer (**Table 3.5**). On ice, cells were removed from the dish mechanically using a clean cell-scraper (Costar) and the lysate was homogenised by repeated dispersion through a pipette tip. The lysate was transferred to a clean 1.5 ml polypropylene tube and microfuged at 350 x **g** for 10 min at 4°C to pellet any cell debris. Supernatants containing whole cell extracts (WCEs) were transferred to a fresh 0.5 ml polypropylene tube and stored at -80°C.

3.5.1.2 Quantification of cellular protein by Bradford Assay.

The protein content of each sample was assessed essentially by the method of Bradford *(313)*. Working out of direct light, 20 µl of sample was added to 1 ml

of protein dye reagent concentrate (Bio-Rad) that had been diluted 20 % v/v with MilliQ-water in a polypropylene tube. BSA stock (10 mg/ ml) was diluted freshly to a concentration of 1 mg/ ml from which ten standards, with protein concentrations between 0 µg/ ml and 20 µg/ ml, were prepared freshly for each plate. Analysing one set of 20 samples at a time, samples and standards were vortexed and 200 µl of each was transferred in duplicate to wells of a 96-well microtitre plate (Nunc). Samples were read immediately at 595 nm in a spectrophotometric plate reader (MR500, Dynatech) and protein concentrations of each were calculated from the standard curve, which plotted protein concentration against OD_{595nm}, using BioLinx 2.10 software (Dynatech Laboratories). Acceptable values for protein concentration of samples were obtained only from plates generating a standard curve with R-square ≥ 0.990. Otherwise, the preparation and analysis of samples and standards were repeated.

3.5.1.3 Protein separation by SDS-PAGE.

Protein separation was carried out based on the method of Laemmli *(314)*. Polyacrylamide (Protogel 37.5:1 Acrylamide: Bisacrylamide, Flowgen) mini-gels were prepared (0.75 mm width; 4% stacking gel, 10 % resolving gel – **Table 3.6**) using Mini Protean II apparatus (BioRad). Once set, gels were submerged in electrode buffer (**Table 3.7**) during sample preparation. An aliquot of broad-range molecular weight marker (BioRad), 10 % v/v, was boiled in 5x SDS sample buffer (**Table 3.6**) and 10 µl added to the first well of each gel. Samples containing 20 µg protein (15-25 µl of lysate) were boiled for 5 min in 5µl of 5x SDS sample buffer in 0.5 ml polypropylene tubes and the total contents of each tube were transferred to the relevant well using gel-loading tips

(Greiner) for accuracy. Samples were electrophoresed at 75 V through the stacking gel and at 200 V through the separating gel until the dye-front was at the very edge of the gel.

3.5.1.4 Immunoblotting.

Electrophoretic transfer of protein onto nitrocellulose membranes was carried based on the method of Towbin *et al. (315)*. The gel containing electrophoresed samples, the nitrocellulose membrane (BDH) and the filter paper (3M, Whatman) all were soaked in transblot buffer (**Table 3.7**) for 10-15 min. After soaking, each gel was laid on top of a nitrocellulose membrane (7.5 x 10 cm). This arrangement was sandwiched between eight sheets of filter paper, which, in turn, was sandwiched between two Scotchbrite pads and clamped into the Mini Transblot Cell cassette (BioRad). Electroblotting was carried out on ice for 1h at 100 V. Transfer of protein onto the nitrocellulose was confirmed by staining the membrane with Ponceau S solution (Sigma). This practice allowed visualisation of molecular weight marker bands, which were marked onto the membrane in pencil. Ponceau S stain was washed away three times with TBS (**Table A4**) + 0.05 % v/v Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and then three times with TBS alone.

3.5.1.5 Detection of protein.

Membranes were blocked overnight in BSA/ ovalbumin blocking buffer (**Table 3.8**) then washed three times in TBS/ tween and three times with TBS alone for 5 min each. Anti-phospho-p44/42 MAPK (ERK) monoclonal antibody (New England Biolabs) was diluted to 1: 1 000 of stock, as recommended by the

manufacturer, with 0.2x blocking buffer in TBS and incubated with the membranes in heat-sealed plastic bags overnight at room temperature with rotation (40 rpm). Membranes were washed, as before, and incubated with HRP-conjugated goat anti-mouse antibody (Dako) diluted to 1: 10,000 of stock, as recommended by the manufacturer, in TBS/ tween for 1 h at room temperature, with rotation (40 rpm). Secondary antibody was washed away and membranes were blotted dry. Each membrane was incubated with chemiluminescence reagent (ECL, Amersham) for 1 min, blotted dry again and wrapped in cling-film. Phosphorylated ERK was detected on the membranes by their exposure to x-ray film (XOMAT, Kodak). Film was processed using a Fuji RGII x-ray film processor.

3.5.1.6 Stripping and re-probing membranes.

Membranes were re-hydrated in TBS before being baked in stripping buffer (**Table 3.6**) for 1 h at 50-55°C. Membranes then were rinsed thoroughly with distilled water and re-blocked for 1 h in BSA/ ovalbumin. After washing three times with TBS/ tween and three times with TBS alone, membranes were incubated with anti-pan ERK antibody (New England Biolabs), diluted 1: 1000 in 0.2x blocking buffer, for 1 h. Membranes were washed as before and incubated with HRP-conjugated goat anti-rabbit antibody (Dako) diluted 1: 10 000 in TBS/ tween for 1 h. Proteins on membranes were visualised as described above.

3.5.2 Toxicity of dicoumarol to pCEP cells.

pCEP cells were seeded into 6-well plates (Nunc) and left to grow for at least 4 days, until almost confluent. Confluent monolayers were washed three times with 2 ml PBS (Gibco) and then cells were bathed overnight in 0.9 ml serum-free DMEM containing only hygromycin and non-essential amino acids. The following day 100 µl of dicoumarol (3,3'-methylene-bis(4-hydroxycoumarin), Sigma) or vehicle control (Milli-Q water, adjusted to pH 9.8) was added to duplicate wells and incubated at 37°C, 5 % CO_2: 95 % air for 30 min plus 3 h, 8 h, 12 h, or 24 h. Alternatively, dicoumarol was left on the cells for 30 min then washed away three times with 2 ml PBS and replaced with 0.9 ml DMEM, containing only hygromycin and non-essential amino acids, for the times indicated. After incubation, cells were harvested using approximately 200 µl trypsin/ EDTA per well. Cell suspensions were centrifuged at 300x **g** for 10 min and viable cells were counted as described previously.

3.6 Analysis of Cytokine Production by Epithelial Cells.

3.6.1 Stimulation of cells.

Cells were seeded into 12-well plates (Falcon) at a density of $1x10^4$/ well or $2x10^4$/ well and grown for 4-7 days until almost confluent. Cell monolayers were washed three times with 1 ml PBS, and 450 µl serum-free medium was replaced into each well for incubation overnight. The following day, 50 µl of vehicle control or agonist were added and cells were incubated for up to 24 h at 37°C in 5 % CO_2: 95 % air. For each inhibitor to be added, 50 µl of serum-free medium were replaced with 50 µl of the specific p38 MAP kinase inhibitor, SB-203580 (4-(4-Fluorophenyl)-2-(4-methylsuphinylphenyl)-5-(4-pyridyl)-1H-

imidazole, Calbiochem) *(316)* or 50 µl of the specific ERK MAP kinase inhibitor PD-098590 (2'-amino-3'-methoxyflavone, Calbiochem) *(142)*. In some experiments, both inhibitors were used together. The plates were wrapped in foil and incubated for 1 h before the addition of agonists. Alternatively, cells were treated with dicoumarol for 30 min and then washed three times with 1 ml PBS. Serum-free medium (450 µl) was replaced and 50 µl agonist was added. SB-203580 and PD-098590 were left in supernatants for the duration of the experiment. Supernatant from each well was collected into 1.5 ml polypropylene tubes, microfuged for 10 min, split into 2 aliquots and stored at -80°C. Microscopic examination after collection of supernatants allowed a gross assessment of whether the cells remained healthy.

3.6.2 Analysis of epithelial cell supernatants for IL-8.

Microtitre 96-well immunoplates (Nunc) were coated with 50 µl/ well of 2 ng/ ml anti-human IL-8 monoclonal antibody (R&D Systems) in PBS, pH 7.4, and incubated overnight at room temperature. Unbound antibody was washed away three times with 200 µl ELISA wash buffer (PBS + 0.05 % tween) per well and the wells were blotted dry. Plates were incubated with 100 µl/ well blocking buffer (**Table 3.9**) for 1 h at room temperature and then washed as before. Samples were prepared in dilution buffer (**Table 3.9**) and 50 µl were added in duplicate to the plate. Standards of nine different concentrations were prepared in dilution buffer from recombinant human IL-8 (R&D Systems), in the range 0-2 ng/ ml. Fifty microlitres per well of each standard was added to each plate in duplicate. Loaded plates were incubated at room temperature for 2 h and then washed. Addition of streptavidin peroxidase (50 µl/ well, 0.5 ng/ ml) was

followed by an incubation period of 30 min at 37°C and further washes. HRP substrate was prepared by addition of OPD (*o*-phenylene-diamine (dihydrochloride), 1 tablet) and H_2O_2 (20 µl of 30 % v/v) to warmed substrate buffer (**Table 3.9**) and was added (100 µl/ well) for incubation at room temperature in the dark for approximately 30 min. The reaction was terminated with 150 µl/ well 1.0 M HCl and $OD_{450\ nm}$ for each well was measured. BioLinx 2.10 software (Dynatech) was used to calculate the IL-8 concentration in each sample by reading values from the standard curve, which plotted IL-8 concentration against $OD_{450\ nm}$.

3.6.3 Selection of antibody concentrations and incubation conditions for IL-10 ELISA.

Two microtitre 96-well immunoplates (Nunc) were coated with either 100 µl/ well or 50 µl/ well of anti-human IL-10 monoclonal antibody (R&D Systems) at either 4 µg/ ml or 2 µg/ ml, in PBS pH 7.4, and incubated overnight at room temperature. Each plate was washed three times with PBS/ tween and 100 µl/ well of blocking buffer was added for incubation for 1 h at room temperature, then the plates were washed as before. IL-10 protein standard (R&D Systems) was diluted in dilution buffer to 0-12 ng/ ml, 50 µl was added to each coated well and one plate was incubated at room temperature for 1 h, whilst the other plate was incubated at 37°C for 1 h. Plates were washed as before and either 100 µl/ well or 50 µl/ well of 250 ng/ ml biotinylated anti-human IL-10 antibody was added to each well. Plates were incubated at 37°C for 1 h then washed three times. Streptavidin peroxidase, OPD/ H_2O_2 and H_2SO_4 were utilised as above and the OD_{490nm} was determined for each well using the same

BioLinx 2.0 software package. Each combination of antibody concentration and sample incubation conditions was assessed in duplicate.

3.6.4 Analysis of epithelial cell supernatants for IL-10.

Microtitre 96-well immunoplates (Nunc) were coated with 100 µl/ well of 2 µg/ ml anti-human IL-10 monoclonal antibody (R&D Systems) in PBS, pH 7.4, and incubated overnight at room temperature. In the same way as for IL-8 detection, plates were washed, blocked and washed again. A range of IL-10 protein concentrations was prepared in dilution buffer and samples were diluted to 20 % v/v in dilution buffer. Samples and standards were loaded onto the plate 50 µl/ well and incubated for 2 h at room temperature. After further washes, 50 µl/ well of 250 ng/ ml biotinylated anti-human IL-10 antibody was added to each well and the plated were incubated for 1 h at 37°C. IL-10 in each well was detected as described previously.

3.7 Statistical Analysis.

Statistical significances were calculated using one-way ANOVA where $n \geq 3$, followed by an appropriate parametric multiple comparison analysis using Dunnett's test for multiple comparisons.

Table 3.1 Collagen gel solution.

Component	Volume
Vitrogen 100 (Cohesion Technologies)	1 ml
DMEM (serum-free)	1 ml
Milli-Q water	13 ml

Table 3.2 Composition of Cl^-- and Cl^--free (NO_3^-) buffers.

Component	Cl^- buffer concentration, mM	NO_3^- buffer concentration, mM
K^+	5.4	5.4
Na^+	130.0	130.0
Ca^{2+}	1.0	1.0
Cl^-	130.0	-
NO_3^-	-	130.0
SO_4^{2-}	3.0	3.0
HPO_4^{2-}	2.4	2.4
$H_2PO_4^{2-}$	0.6	0.6
Glucose	10.0	10.0
Mg^{2+}	1.0	1.0
HEPES, pH7.4	10.0	10.0

Table 3.3 Efflux buffer for radioisotopic analysis.

Component	Concentration, mM
NaCl	140
KH_2PO_4	3.3
K_2HPO_4	0.83
$CaSO_4$	1
$MgSO_4$	1
Glucose	10
HEPES, pH 7.4	10

Table 3.4 Wash buffer for radioisotopic analysis.

Component	Concentration, mM
$MgCl_2$	137.0
Sucrose	100.0
HEPES, pH 7.4	50.0

Table 3.5 Lysis buffer for WCE collection.

Component	Final concentration
Tris HCl, pH 7.5	50 mM
NaCl	150 mM
NP40 (Nonidet P40) (BDH/Merck)	1 % v/v
Glycerol	10 % v/v
EDTA, pH 8.0	5 mM
Na_3VO_4 (Calbiochem)	1 mM
$NaMoO_4$	1 mM
NaF	10 mM
PMSF (phenylmethylsulphonyl-fluoride)	40 µg/ ml
Pepstatin A	0.7 µg/ ml
Aprotinin	10 µg/ ml
Leupeptin	10 µg/ ml
STI (soybean trypsin inhibitor)	10 µg/ ml

Table 3.6 Composition of polyacrylamide gels and buffers for SDS-PAGE.

Component	Stacking gel (4%)	Resolving gel (10%)	Sample buffer (5x)	Stripping buffer
Acrylamide/ Bis soln.	1.33 ml	5.0 ml	-	-
SDS (10%w/v)	0.15 ml	0.25 ml	5 g	20 ml
Tris pH 8.8 (1M)	-	5.6 ml	-	-
Tris pH 6.8 (1M)	1.25 ml	-	10 ml	8 ml
Milli-Q	6.36 ml	4.35 ml	11.5 ml	72 ml
TEMED soln (10%)	20 µl	20 µl	-	-
AMPS (10% w/v)	50 µl	50 µl	-	-
Glycerol	-	-	25 ml	-
BPB soln. (5 % w/v)	-	-	400 µl	-
2-ME	-	-	2.5 ml	770 µl

Abbreviations: TEMED - N,N,N,'N'-tetramethylethylene diamine; AMPS – ammonium persulphate (prepared freshly each week); BPB – bromophenol blue; 2-ME – 2-mercaptoethanol.

Table 3.7 Composition of electrophoresis buffers for SDS-PAGE.

Component	Electrode buffer	Transblot buffer
Tris HCl	3.0 g	3.0 g
Glycine	14.4 g	14.4 g
SDS (10 % w/v)	10 ml	-
Methanol	-	200 ml
Milli-Q	to 1 L	to 1 L

Table 3.8 Composition of blocking buffer for Western blotting.

Component	Quantity
BSA	5 g
Ovalbumin	1 g
NaN_3	0.01 g
Milli-Q	to 100 ml

Table 3.9 Composition of ELISA buffers.

Component	Blocking buffer	Dilution Buffer	Substrate Buffer
BSA	10 g	0.1 g	-
Sucrose	50 g	-	-
NaN_3	0.5 g	-	-
Citric acid (monohydrate)	-	-	7.3 g
Na_2HPO_4	-	-	9.46 g
Tween-20	-	50 µl	-
TBS	-	to 100 ml	-
Milli-Q	to 1 L	-	to 1 L

CHAPTER 4:

Modulation of chloride channel activity in the respiratory epithelium

4.1 Introduction.

CF is characterised by an abnormal electrolyte balance across the epithelial mucosae because of defective chloride transport through CFTR. Many bacterial species, including *E. coli (19;317;318)* and *P. aeruginosa (319-321)*, have been shown to affect ion channel activity, although bacterial modulation of Cl^- transport in the lung has not been demonstrated directly. This chapter describes a series of experiments that were designed to investigate the possibility that clinically significant CF pathogens, *P. aeruginosa* and *B. cepacia* modulate Cl^- movement across the respiratory epithelium and if so, whether any differences exist between the Cl^- channel responses in CF and non-CF epithelial cells.

Chloride channel activity was measured in respiratory epithelial cell lines that had been stimulated either with pharmacological mediators, or with *P. aeruginosa* PAO1 or *B. cepacia* J2315. A range of different techniques was used, employing either fluorescent or radioisotopic chloride probes, and several chloride channel agonists served as positive controls in these assays. These agonists are described in **Table 4.1.**

Table 4.1 Pharmacological actions of agonists used to investigate Cl^- efflux.

Agonist	Mode of action	Immediate response	Cellular effects	Expected effect on Cl^- efflux from each cell phenotype
Ionomycin	Calcium ionophore	Sequestered Ca^{2+} released from intracellular stores Influx of extracellular Ca^{2+}	Increased Ca^{2+} concentration activates CaMCC [a]	**CF** - Increased efflux **non-CF** - Increased efflux
Isoprenaline	β-adrenergic receptor agonist	cAMP production increased	Activation of cAMP-mediated Cl^- channel (CFTR)	**CF** - No increase **normal** - Increased efflux
8-Br-cAMP	Non-hydrolysable analogue of cAMP	High levels of cAMP maintained	Activation of cAMP-mediated Cl^- channel (CFTR)	**CF** - No increase **normal** - Increased efflux
Forskolin	Activates adenylate cyclase	cAMP production increased	Activation of cAMP-mediated Cl^- channel (CFTR)	**CF** - No increase **normal** - Increased efflux
IBMX (Calbiochem)	Non-specific PDE inhibitor	High levels of cAMP maintained	Activation of cAMP-mediated Cl^- channel (CFTR)	**CF** - No increase **normal** - Increased efflux

[a] Abbreviations: CaMCC – calcium-mediated Cl^- channel; CF – cells expressing CF mutation; cAMP – cyclic AMP; 8-Br-cAMP - 8 bromo cyclic AMP; IBMX – 3-isobutyl-1-methylxanthine; PDE - phosphodiesterase

4.2 Investigation of Cl^- Channel Activity Using the Radioisotope ^{36}Cl.

Epithelial cells were grown in 35 mm-petri dishes and loaded with ^{36}Cl for up to 5 h *(308)*. Unloaded radioactivity was washed away and cells were overlaid with efflux buffer. Supernatant was collected every minute before and after the addition of agonist for up to 10 min. Changes in radioactivity in the cell supernatant over time were analysed after the addition of a cAMP agonist (isoprenaline, forskolin + IBMX, or 8Br-cAMP) or addition of a calcium ionophore (ionomycin) and these data were compared with unstimulated cells and buffer control. Only ionomycin (10 μM) increased the efflux of ^{36}Cl from stimulated epithelial cells but no difference could be detected between the level of radioactivity in the supernatant from unstimulated cells and that from cells treated with a cAMP agonist (data not shown). This assay displayed low reproducibility because often, baseline efflux was very high, masking any effects that there may have been with the Cl^- channel agonists.

It has been reported that fluorescent probes are more sensitive that radioisotopic probes *(322)*, as well as being non-toxic. To improve the sensitivity of the assay, and to avoid the potential hazards associated with radiolabelled chemicals, a fluorescent Cl^- sensitive dye was tested in subsequent experiments.

4.3 Analysis of Epithelial Cl^- Transport Using a Fluorescent Probe.

A number of groups have used fluorescent indicators to assess Cl^- transport in a variety of epithelial cell types *(310;323-325)*. MQAE is a Cl^--sensitive dye,

whose fluorescence is reduced by collision with halide ions in a concentration-dependent fashion. The sensitivity of fluorescent probes is described by the Stern-Volmer quenching constant, K_{SV}, which describes the reciprocal of the ion concentration that quenches 50 % of the maximum fluorescence. Developed by Verkman and colleagues *(322;326)*, MQAE was selected because it can be loaded into the cells non-invasively, and because it is more sensitive (K_{SV} = 200 M^{-1}) than another commonly used Cl^- probe, SPQ (K_{SV} = 118 M^{-1}). However, both dyes are less sensitive in cells (MQAE K_{SV} = 64 M^{-1}; SPQ K_{SV} = 12 M^{-1}) than in solution. MQAE was loaded into epithelial cells and measured using three different fluorimetric systems, and procedures adapted from several published methods *(326-329)*.

4.3.1 Fluorescence measurement of Cl^- transport in epithelial cells using a cuvette system.

CFTE and HTE cells were grown in T25 flasks and loaded with MQAE as required. Harvested epithelial cells were washed and suspended in Cl^- free buffer so that Cl^- could move out of the cell down a concentration gradient once the appropriate channels were opened. The extracellular anionic balance was restored with NO_3^-, which permeates Cl^- channels readily but it does not quench MQAE fluorescence. The principle of this model is depicted in **Figure 3.1**.

Epithelial cells were stimulated with ionomycin, isoprenaline or heat-killed *B. cepacia* J2315, and the fluorescence of each sample was measured over time. The results are displayed graphically (**Figure 4.1**) and are shown in **Table 4.2**. Percentage change in fluorescence, ΔF, was calculated as:

$$\Delta F = \left((F_t - F_{min}) / (F_{max} - F_{min})\right) \times 100\ \%$$

where F_t is the fluorescence at time, t; and the degree of dye loading was calculated as the difference between F_{min} and F_{max}.

4.3.1.1 Results from MQAE cuvette assay.

Heat-killed *B. cepacia* J2315 seemed to increase the fluorescence of MQAE-loaded HTE cells (**Figure 4.1 A**) and CFTE cells (**Figure 4.1 B**) over a 5 min period, compared with unstimulated cells (**Table 4.2**), although these differences were not significant by ANOVA analysis. Only CFTE cells stimulated with 10 μM ionomycin for 5 min showed a significant increase in MQAE-fluorescence compared with unstimulated cells (ANOVA, $p<0.05$). *B. cepacia* J2315 may activate a transepithelial Cl^- current in CF airway epithelial cells, but this stimulation would need to be repeated at least once to be sure, since only n=2 data were obtained for this stimulation using this technique.

Ionomycin, but not cAMP agonists, increased the MQAE-fluorescence of CFTE cells (**Table 4.2; Figure 4.1 B**), which is consistent with reports that Ca^{2+} ionophore-stimulation of CFTE cells induces Cl^- efflux and lack functional CFTR *(327)*. No response was seen in HTE cells to either class of agonists, despite the fact that these cells should respond to both. These caveats may be the result of physiological instabilities of adherent cells kept in suspension, which create high background fluorescence because of increasing membrane permeability. This point was addressed in the next set of experiments. Since epithelial cells do not grow in suspension *in vivo* and thus may not behave in

suspension as they would when in an adherent monolayer, subsequent studies focused on analysing transepithelial Cl^- flux in adherent epithelial cultures.

4.3.2 Fluorimetric analysis of cells grown on coverslips.

CFTE, HTE or pCEP cells were grown on coverslips and loaded with MQAE *(310)*. A small island of epithelial cells was brought into focus. Fluorescence was measured first in Cl^- buffer and then in NO_3^- buffer, before addition of the agonist. Unavoidable disturbances in the fluorescence signal caused by manual aspiration of buffer in the dark, between measurements, affected the reproducibility of this assay (data not shown). Therefore, an alternative method for measuring MQAE fluorescence in adherent epithelial cells was adopted, employing a semi-automated fluorimetric system.

4.3.3 Fluorimetric analysis of Cl^- transport using a semi-automated plate-reader.

Epithelial cell lines were grown in 8-well strips of a microtitre plate and loaded with MQAE. Baseline cellular fluorescence was measured in the presence and then the absence of Cl^- as before, using a Fluoroskan Ascent plate reader. Adherent epithelial cells were stimulated with pharmacological agonists or with fresh stationary phase cultures of *P. aeruginosa* PAO1 or *B. cepacia* J2315 (moi = 100).

It was found that the presence of *P. aeruginosa* PAO1 supernatant almost doubled the change in fluorescence that was detected. The difference between the fluorescence generated using whole cell suspension PAO1 and supernatant-

free PAO1 is shown in **Figure 4.2.** Because some *P. aeruginosa* exoproducts (for example pyoverdin and pyocyanin) fluoresce at a similar wavelength to MQAE they interfere with the fluorescence signal, cultures of *P. aeruginosa* PAO1 were pelleted then suspended in M9 and diluted in NO_3^- buffer for stimulating epithelial cells in this assay.

4.3.3.1 Results from Fluoroskan Ascent plate reader assay with MQAE

Data from this set of experiments are detailed in **Table 4.3**, and illustrated in **Figure 4.3**. *P. aeruginosa* PAO1 enhanced MQAE fluorescence in both CF and normal epithelial cells, compared with negative controls. In CFTE cells, PAO1 elicited more than a 7-fold increase in Cl^- efflux after 1 min (ANOVA, $p<0.01$), which was sustained over the 5 min period (ANOVA, $p<0.05$) elevating the fluorescence to approximately 40 % of the maximum **(Figure 4.3 B)**. This effect was repeated in the second CF cell line, pCEP-RF **(Figure 4.3 D)**, in which PAO1 induced a significantly enhanced transepithelial Cl^- current compared with buffer alone (ANOVA, $p<0.05$), raising fluorescence to approximately 50 % of the maximum. An even greater effect was seen in pCEP-2 cells stimulated with *P. aeruginosa*, which demonstrated fluorescence of almost 70 % of the maximum when Cl^- efflux was elevated significantly (ANOVA, $p<0.001$) compared with control cells for the duration of the experiment **(Figure 4.3 C)**. After 1 min of stimulation with PAO1, pCEP-2 fluorescence had increased 12-fold over control. No significant effect was observed in HTE cells (ANOVA, $p>0.05$).

Furthermore, **Figures 4.3 C and 4.3 B**, respectively, show that the epidemic *B. cepacia* strain, J2315, activated sustained Cl^- efflux both from pCEP-2 cells (ANOVA, $p<0.001$) and from CFTE cells (ANOVA, $p<0.01$) within 1 min of stimulation, and whilst *B. cepacia* elevated the fluorescence of pCEP-RF cells **(Figure 4.3 D)**, this increase was not considered to be significant (ANOVA, $p>0.05$). These results suggest that both *P. aeruginosa* and *B. cepacia* activate Cl^- channels in respiratory epithelial cells. Although the fluorescent signal continued to rise in intensity over the 5 min period in all samples, the initial rates of increase were not sustained but they began to reach a steady state.

Activation of Cl^- channels in CFTE cells was more pronounced after bacterial stimulation than after treatment with ionomycin ($p_{(1min)} < 0.01$; $p_{(5min)} <0.05$), and a similar situation was seen in pCEP-2 cells (**Figure 4.3 B-C**). *B. cepacia* generated a stronger Cl^- efflux from pCEP-2 cells than did ionomycin after 5 min (ANOVA, $p<0.05$) and *P. aeruginosa* treatment was significantly and consistently more effective at eliciting Cl^- currents in this cell line (ANOVA, $p<0.001$).

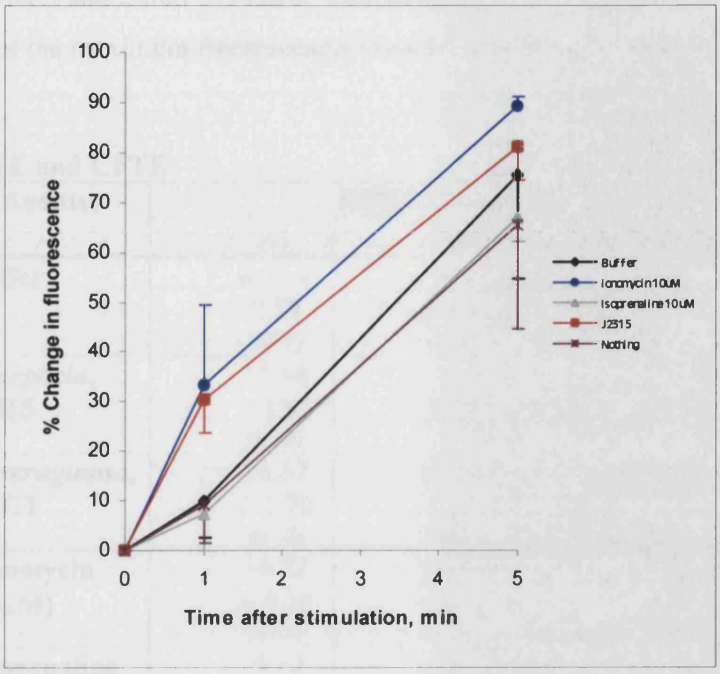
Bacterial stimulation of CFTE cells generated a similar elevation in fluorescence intensity regardless of the strain ($p_{(1min)}>0.48$; $p_{(5min)}>0.84$). However, the response of pCEP-2 cells to *P. aeruginosa* was even greater than to *B. cepacia* (ANOVA, $p<0.001$). Taken together, these observations imply that whilst *P. aeruginosa* and *B. cepacia* activate Cl^- channels in respiratory epithelial cells, rates of Cl^- efflux vary according to the epithelial and bacterial phenotype.

Table 4.2 Effects of *B. cepacia* J2315 and other agonists on the fluorescence of MQAE in suspension epithelial cells, measured with a PTI fluorimeter. Changes in fluorescence 1 min (Δ1) and 5 min (Δ5) after stimulation are expressed as a percentage +/- SEM of the maximum fluorescence of each sample.

Agonist	HTE		CFTE	
	Δ1	Δ5	Δ1	Δ5
Nothing	9.15 ± 7.58 *(n=4)*	65.54 ± 20.80 *(n=4)*	11.95 ± 1.54 *(n=7)*	39.99 ± 8.05 *(n=7)*
Buffer	10.15 ± 6.25 *(n=3)*	75.55 ± 16.53 *(n=3)*	13.81 ± 6.01 *(n=5)*	45.55 ± 11.70 *(n=5)*
***B. cepacia*, J2315**	30.57 ± 6.89 *(n=3)*	81.04 ± 6.76 *(n=3)*	18.35 *(n=2)*	64.46 *(n=2)*
Ionomycin (10μM)	33.58 ± 15.73 *(n=4)*	89.25 ± 1.96 *(n=4)*	16.38 ± 5.31 *(n=6)*	80.37 ± 8.20 *(n=6)*
Isoprenaline (10μM)	7.47 ± 7.33 *(n=3)*	66.95 ± 5.00 *(n=3)*	7.35 ± 2.70 *(n=6)*	51.96 ± 9.59 *(n=6)*
Forskolin (10μM) + IBMX (10μM)	9.76 ± 4.94 *(n=4)*	59.63 ± 23.29 *(n=4)*	12.95 ± 4.12 *(n=4)*	58.03 ± 9.13 *(n=4)*

Figure 4.1 (next page) MQAE fluorescence of epithelial cell suspensions, measured with a PTI fluorimeter. Error bars (SEM) that are not visible were smaller than the data marker.

A: HTE


100
90
80
70
60
50
40
30
20
10
0
% Change in fluorescence
0
1
2
3
4
5
Time after stimulation, min
Buffer
Ionomycin10uM
Isoprenaline10uM
J2315
Nothing

B: CFTE

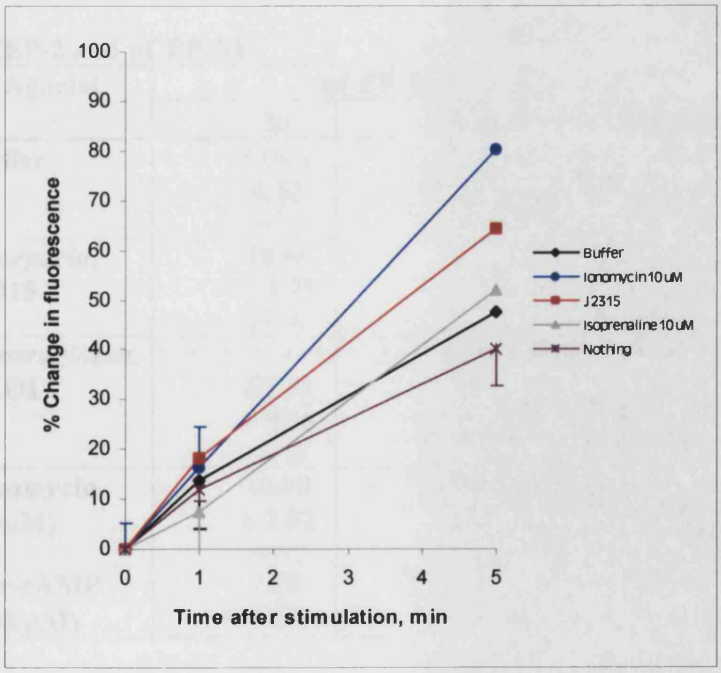

100
90
80
70
60
50
40
30
20
10
0
% Change in fluorescence
0
1
2
3
4
5
Time after stimulation, min
Buffer
Ionomycin10uM
J2315
Isoprenaline10uM
Nothing

Table 4.3 Effects of bacteria on the fluorescence of MQAE in adherent epithelial cells, measured with Fluoroskan Ascent. Changes in fluorescence 1 min (Δ1) and 5 min (Δ5) after stimulation are expressed as a percentage +/- SEM of the maximum fluorescence of each sample.

A: HTE and CFTE

Agonist	HTE		CFTE	
	Δ1	Δ5	Δ1	Δ5
Buffer	8.52 ± 2.59 *(n=4)*	11.77 ± 2.38 *(n=4)*	4.7 ± 8.54 *(n=4)*	9.12 ± 10.64 *(n=4)*
***B. cepacia*, J2315**	15.86 ± 1.65 *(n=3)*	23.27 ± 3.21 *(n=3)*	30.05 ± 2.68 *(n=3)*	37.94 ± 5.80 *(n=3)*
***P. aeruginosa*, PAO1**	21.57 ± 1.70 *(n=3)*	31.25 ± 4.34 *(n=3)*	32.91 ± 2.56 *(n=3)*	39.63 ± 5.91 *(n=3)*
Ionomycin (10μM)	14.22 ± 4.30 *(n=3)*	23.20 ± 2.70 *(n=3)*	6.30 ± 0.74 *(n=3)*	12.86 ± 1.33 *(n=6)*
Isoprenaline (10μM)	-5.62 *(n=2)*	-2.66 *(n=2)*	1.76 *(n=2)*	4.53 *(n=2)*
8Br-cAMP (250 μM)	8.78 ± 11.42 *(n=3)*	13.54 ± 9.74 *(n=3)*	6.19 ± 1.85 *(n=3)*	11.33 ± 1.44 *(n=3)*

B: pCEP-2 and pCEP-RF

Agonist	pCEP-2		pCEP-RF	
	Δ1	Δ5	Δ1	Δ5
Buffer	5.09 ± 4.52 *(n=3)*	13.14 ± 3.03 *(n=3)*	12.28 ± 4.14 *(n=3)*	24.24 ± 2.41 *(n=3)*
***B. cepacia*, J2315**	18.66 ± 3.71 *(n=4)*	35.82 ± 4.70 *(n=4)*	23.16 ± 9.20 *(n=3)*	31.32 ± 9.07 *(n=3)*
***P. aeruginosa*, PAO1**	62.09 ± 8.28 *(n=4)*	70.92 ± 4.12 *(n=4)*	52.56 ± 11.95 *(n=3)*	53.47 ± 13.79 *(n=3)*
Ionomycin (10μM)	10.10 ± 2.03 *(n=3)*	21.16 ± 1.63 *(n=3)*	12.88 ± 9.03 *(n=3)*	31.48 ± 3.49 *(n=3)*
8Br-cAMP (250 μM)	1.98 *(n=2)*	4.46 *(n=2)*	0.57 *(n=2)*	9.89 *(n=2)*

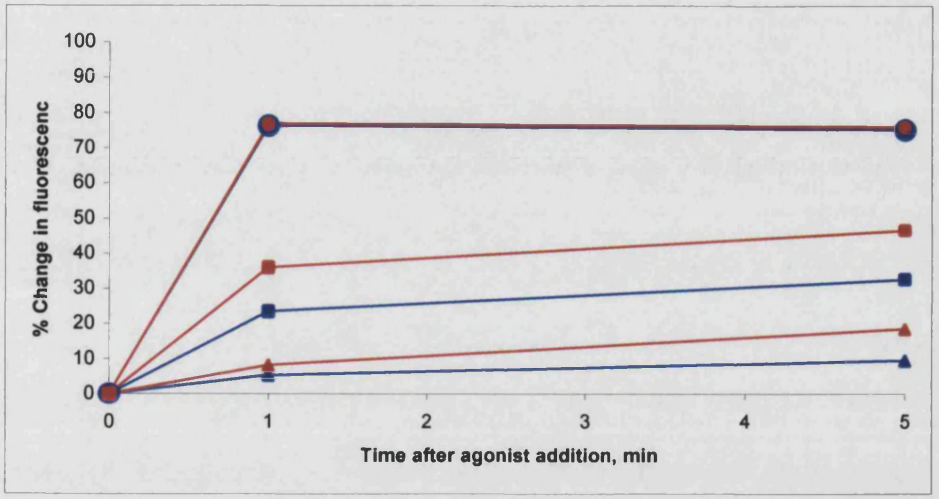


Figure 4.2 Interference of *P. aeruginosa* PAO1 supernatant with MQAE fluorescence, measured with plate-reader. HTE (blue); CFTE (red). Triangle – buffer; square – *Pseudomonas* supernatant; circle- *Pseudomonas* whole culture. (n=1)

Figure 4.3 (next two pages) Effect of PAO1 and J2315 on MQAE-fluorescence of adherent respiratory epithelial cells measured with Fluoroskan Ascent plate reader. Error bars represent SEM for each sample and where not visible, error bars were smaller than the data point.

Figure 4.3 A: HTE

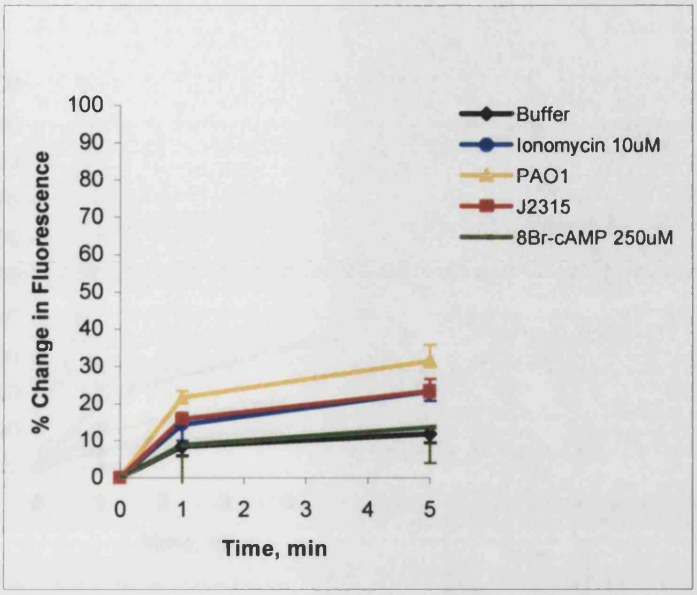


Figure 4.3 B: CFTE

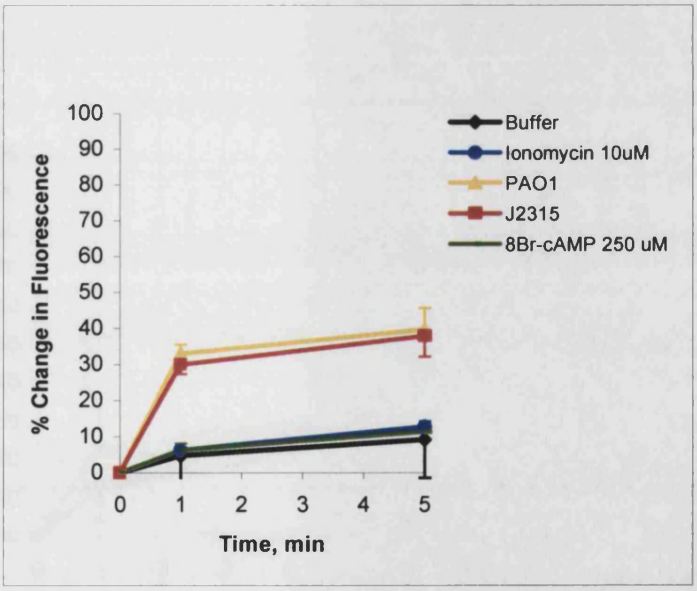


Figure 4.3 C: pCEP-2

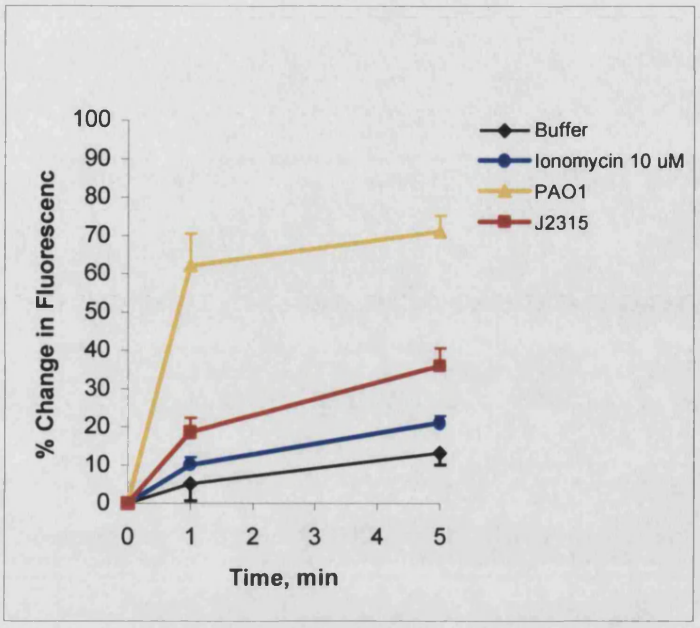
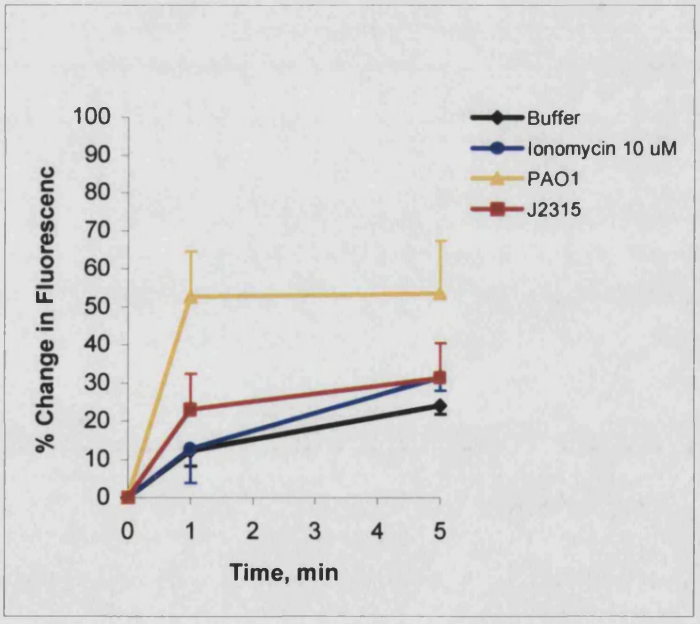


Figure 4.3 D: pCEP-RF



4.4 Discussion.

Bacterial challenge within the respiratory tract activates a plethora of signalling pathways in the host cell that commonly are mediated by second messengers. These small diffusible signalling molecules include Ca^{2+} and cyclic nucleotides and they regulate a number of important cellular processes such as the activity of membrane ion channels. The major physiological manifestation of CF is a disturbed electrolyte balance at epithelial mucosae because of reduced functional expression of the epithelial Cl^- channel, CFTR. Abnormal electrolyte movement dehydrates the overlying mucous blanket and the mucociliary escalator is disabled. Highly adapted bacterial pathogens such as *P. aeruginosa* and *B. cepacia* can withstand this hostile, nutrient replete environment to cause repeated and persistent infections that contribute significantly to the pathophysiology of the CF lung. The aim of the work presented in this chapter was to determine whether these pathogens might modulate Cl^- channels within the lung, and contribute to this electrolytic disruption.

Using bovine tissue and a capacitance probe technique, Evans *et al.* demonstrated recently that *P. aeruginosa* altered the airway fluid transport in bovine tissue, and that this was most likely via effects on basolateral K^+ channels and apical Na^+ channels in the respiratory epithelium *(319)*. This study also suggested that *P. aeruginosa* may modulate other ion transport processes, including anionic channels. Since Cl^- is the predominant cellular anion, it is likely that their observations could translate into an activation of Cl^- transport by *P. aeruginosa*. Other research groups have shown that a secreted heat-stable *E. coli* enterotoxin hyper-activates CFTR specifically in the gut, by

the direct activation of cAMP and cGMP production *(19;196;317;330)*, resulting in enhanced transepithelial Cl^- transport that causes a massive and constant outpouring of watery stools *in vivo*. This process provides *E. coli* with a mechanism of dispersal to the next host to ensure continuation of the infection. Similarly, *Vibrio parahaemolyticus*, which is another important cause of gastroenteritis, produces a haemolytic toxin that activates CaMCC in human colonic epithelial cells *(331)*. Thus, bacterial interference with electrogenic processes in epithelia is an important and well-documented mechanism of pathogenesis and may be important in CF lung infections. In view of the pleiotropic effects of CFTR in the cell, this molecule would not be an unexpected target for bacterial disruption of host signalling pathways and potential diversion or inactivation of the immune response in the lung.

The primary aim of this chapter was to develop a suitable experimental system to assess the effects that *P. aeruginosa* or *B. cepacia* may have on transepithelial Cl^- currents in the respiratory epithelium and whether any such effects may be mediated by CFTR. Numerous techniques are available for the investigation of ion channel activity in epithelial cells, including the use of radioactive or fluorescent intracellular probes. Both of these methods were tested here for use with the immortalized CF and non-CF epithelial cells.

4.4.1 Development of a sensitive and reproducible assay to measure Cl^- efflux in respiratory epithelial cells *in vitro*.

^{36}Cl efflux was measured in HTE cells and CFTE cells that had been treated either with the calcium ionophore ionomycin, which activates Ca^{2+}-mediated Cl^-

channels (CaMCC); or with a cAMP-agonist such as isoprenaline, which activates adenylate cyclase via its G-protein coupled receptor and activates CFTR via PKA phosphorylation. As expected, ionomycin elicited Cl^- efflux from both the non-CF and CF-phenotype cell lines that was higher than background fluctuations. This was consistent with published reports that both pairs of cell lines express Ca^{2+}-mediated Cl^- channels *(307;308;310)*. However, it was not possible to demonstrate a clear difference between the response of CFTE cells and that of HTE cells to isoproterenol using ^{36}Cl despite the fact that the double ΔF508 mutation renders CFTE unresponsive to cAMP agonists *(308)*, whilst HTE cells retain Cl^- transport activity through CFTR *(307)*. At this point it is of interest to note that whilst studies in the laboratory from which both of these cell lines originate employed both electrophysiological and radioactive tracer techniques to report the Cl^- transport properties of CFTE cells and to demonstrate a lack of responsiveness to cAMP agonists *(308)*, only electrophysiological demonstration of HTE Cl^- transport was provided *(307)*. Thus, CFTR-mediated Cl^- currents had not been demonstrated previously in HTE cells using radioisotopic probes. There was high background noise in this system, which hindered the reproducibility of this assay and made it unsuitable in this situation. Bacteria were not tested in this system.

Subsequent experimental systems involved a fluorescence Cl^- probe, MQAE, since it has been reported that fluorimetric techniques are more sensitive that assays with radioisotopes *(322)*. Furthermore, use of these non-toxic fluorescent dyes circumvents the potential hazards associated with measurement of radioisotopic tracers. In the first instance, MQAE fluorescence was

measured in epithelial cells in suspension in a cuvette so that the cellular concentration could be adjusted easily to optimise the signal, whilst a magnetic stirrer in the bottom of the cuvette ensured even distribution of cells and agonists. In addition, this procedure permitted access to the basolateral epithelial membrane, which is likely to occur *in vivo* in the event of inflammation or tissue damage. To maximise any change in fluorescence, Cl^- ions were removed from the extracellular fluid so that Cl^- would flow out of the cell, and consequently the fluorescence intensity would increase, should the appropriate channels be activated, as depicted schematically in **Figure 3.1**. A marked change in fluorescence was detected in stimulated cells, although unstimulated epithelial cells in suspension also displayed increased MQAE-fluorescence over the 5 min duration of the experiment. Adherent cells lose physiological stability with time when in suspension, which may have contributed to the 'leakiness' of the epithelial cells so the protocol was adapted to measure cells growing on a substrate.

To begin with, epithelial cells were grown on glass coverslips and MQAE fluorescence was measured in response to Cl^- channel agonists using a PTI fluorimeter. Experiments were performed with and without extracellular Cl^- but neither approach generated a particularly strong signal. Thus, for a number of reasons, the most useful fluorescence-based system with which to monitor epithelial Cl^- currents was the semi-automated Fluoroskan Ascent plate reader. Firstly, Cl^- efflux could be measured in adherent epithelial monolayers, which reduced the baseline efflux of unstimulated cells that was seen in the cuvette system quite considerably (compare **Figure 4.1 A** with **Figure 4.3 A**, for

example). In addition, multiple replicates could be performed for each sample, providing a more accurate representation of the data. Finally, in contrast to the coverslip assay, samples could be mixed thoroughly and signals were detected with relatively small numbers of cells.

4.4.2 Bacterial modulation of Cl^- channels in airway epithelial cells.

Both *P. aeruginosa* and *B. cepacia*, two important pathogens associated with CF pulmonary infection displayed the potential to interfere with Cl^- channel activity *in vitro*. For example, *P. aeruginosa* PAO1 increased the fluorescence signal from the CF-phenotype cell lines CFTE and pCEP-RF, as well as from the normal pCEP-2 cells (**Figure 4.3 B-D**, respectively). Possible candidates for the activated Cl^- channel include cAMP-activated CFTR; ORCC, which is regulated by CFTR expression and cell swelling *(46;332)*; and CaMCC that is activated by ionomycin *(333)*. It has been reported that pCEP-RF and CFTE cells do not express functional CFTR activity and CFTE cells may exhibit lower activity of CaMCC, whilst pCEP-2 cells retain sensitivity towards agonists of the cAMP pathway *(308;310)*. In order to determine precisely the identity of the channel targeted by PAO1, each Cl^- current would need to be inhibited specifically and selectively. Since there appears to a slight difference between the efflux rates in CF and non-CF epithelial cells stimulated with *P. aeruginosa* PAO1, but this difference just failed to be significant (ANOVA, $p<0.074$), the possibility that more than one group of Cl^- channels is activated in response to *P. aeruginosa* infection should not be ruled out.

Three important features of the results presented here are in agreement with the report that *P. aeruginosa* induces fluid secretion at the respiratory epithelium. To begin with, the data presented here show that PAO1 activates Cl^- currents in epithelial cells, which was implied by Evans *et al.* with the use of Cl^- channel inhibitors that blocked the effects of *P. aeruginosa* on fluid absorption in their electrophysiological assay. Secondly, in none of the Cl^- transport methods described here was it possible to detect the effects of cAMP agonists on Cl^- transport, yet strong signals were obtained in response to bacterial stimulation. This discrepancy is consistent with the idea that a channel other than CFTR was activated although this evidence is circumstantial and does not exclude a role for CFTR. Evans *et al.* showed the effects of *P. aeruginosa* on fluid movement to be abrogated by the Cl^- channel inhibitors H_2-DIDS and NPPB yet the inhibitory potential of these compounds against airway CFTR channel activity is disputed *(332;334-336)*. Again, the implication is that CFTR is not involved in bacterial-induced Cl^- currents in epithelial cells. Finally, because the PAO1 pigments secreted under iron-limited growth conditions fluoresce at a similar wavelength to that of MQAE so that PAO1 supernatant may interfere with the MQAE signal, stimulations were performed with supernatant-free cultures of *P. aeruginosa.*

Therefore, it can be concluded from this result that the Cl^- current measured fluorescently was not induced by a secreted *P. aeruginosa* component, but required the presence of bacterial cells. In the same way, the electrophysiological studies had shown that the altered epithelial fluid transport was dependent upon cell-to-cell contact between *P. aeruginosa* and the bovine

epithelium, since mutants defective in expression of RpoN did not generate the same response in bovine tracheal epithelial cells as the wild-type *P. aeruginosa* cultures *(319)*. RpoN is an alternative sigma factor of RNA polymerase that is required for the expression of certain bacterial adhesins *(337)*. This observation suggests either that a direct and specific bacterial interaction with a host cell surface receptor is required to initiate the enhanced fluid secretion, or that a toxin secreted via the cell-to-cell contact dependent Type III pathway may be involved.

To date, no other studies have examined the potential effects of *B. cepacia* on the electrolytic composition of airway surface fluid, despite the clear relevance that such a finding would have for CF lung disease. The results presented here show that an epidemic strain of *B. cepacia* activated Cl^- channels in normal and CF airway cells, independently of CFTR. Using the cuvette model, strain J2315 generated a Cl^- current rapidly in HTE cells that followed closely the pattern of ionomycin-induced fluorescence, which could suggest CaMCC as a target of *B. cepacia*. However, the initial rate of 30 % efflux per min was not sustained and after 5 min, the fluorescence traces generated by ionomycin and J2315 began to diverge. Thus whilst *B. cepacia* elicited Cl^- efflux in both CF and normal tracheal epithelial cells, the kinetics of these currents appeared to differ slightly between the two cell lines. This potential discrepancy was investigated more closely using the Fluoroskan Ascent system.

B. cepacia J2315 activated Cl^- currents in both CFTE (ANOVA, $p<0.01$) and pCEP-2 cells (ANOVA, $p<0.01$), but no significant change was seen in either

HTE or pCEP-RF cells in response to this pathogen. Cl^- channel activity was induced within 1 min and sustained in CFTE cells, but the difference was only detectable in pCEP-2 cells after 5 min. This caveat would support the hypothesis that the Cl^- currents in response to *B. cepacia* display different kinetics in CF and normal cells, suggesting that different Cl^- channels may be involved in each cell line, although the overall effects after 5 min show no difference between the increased fluorescence in CFTE and pCEP-2 cells (ANOVA, $p>0.78$).

It is difficult to speculate about the molecular interactions that are important for these bacterial-induced Cl^- currents. Indeed, more than one family of anion channels may be involved, and the potential involvement of CFTR cannot be excluded. Activation of CaMCC would require an intracellular Ca^{2+} flux, which could be measured fluorescently. Alternatively, a selective inhibitor of CaMCC and ORCC, such as H_2-DIDS *(331)* could be used to assess the contribution of these channels to bacterial-induced Cl^- currents in airway epithelial cells. Similarly, specific and selective inhibition of CFTR using glibenclamide *(331)* might determine whether this channel conducts Cl^- currents as a result of stimulation with *B. cepacia* or *P. aeruginosa.* In view of the inconsistencies surrounding channel blockers, an alternative approach would be to examine the effects of these bacteria in A549 lung epithelial cells, which are known not to express CFTR mRNA *(338).* A requirement for cell-to-cell contact between *P. aeruginosa* and the host cell has been demonstrated modulation of epithelial fluid transport *(319).* To elucidate the bacterial factors that are important for the observed effects on epithelial Cl^- transport, it would be of great interest to test

bacterial mutants, such as the Type III secretion system mutants of *P. aeruginosa* PAK strain in the fluorimetric system that was described in this chapter. In this respect, supernatant-free cultures of *B. cepacia* J2315, could be examined to identify whether secreted factors may be important in the instigation of Cl^- efflux at the respiratory epithelium, since the pathways of channel activation may differ between these two CF pathogens.

Cl^- is the most abundant intracellular and extracellular anionic species in most tissues and, as such, is pivotal in regulating physiological parameters such as cell volume, pH, and secretion and reabsorption of salt and fluid across the epithelial membrane. Exaggerated transepithelial Cl^- currents could confer a survival advantage upon bacterial pathogens in a number of ways, which would enhance pulmonary infection in the CF lung. Currently there remains considerable debate about the precise effects of *CFTR* mutations on the tonicity and hydration of ASF in the CF lung compared with normal tissue, partly as a consequence of the very low yield of ASF obtainable for *in vitro* studies. For example, at least two different groups have reported that there is no difference between the salinity of ASF in normal tissue compared with CF *(61;339)*, although the exact values for Na^+ and Cl^- concentration differed substantially in each case. Matsui *et al.* proposed that the distinction between CF and normal ASF concerned the volume rather than the salinity of ASF *(61)*. Based on the model of hyperabsorption of fluid by the respiratory epithelium in CF, these researchers state that reduced ASF volume prevented the disengagement of the overlying blanket of mucus by the epithelial cilia during the ratchet process of mucociliary clearance. Equally, if the ASF were too deep, because of excessive

Cl^- and fluid secretion, the protruding cilia would be unable to engage the mucous blanket, again preventing the removal of aspirated and ensnared bacteria by the mucociliary escalator.

An alternative hypothesis arose from an illuminating study by Smith and colleagues *(69)*, who provided compelling evidence that Na^+ and Cl^- concentrations were elevated in CF airways and displayed reduced antibacterial activity compared with normal tissue, which could be reversed by restoring normal salinity to the CF cell supernatant. Thus, disturbed electrolyte balance may enhance bacterial survival in the CF lung by inactivating salt-sensitive components of the immune system, such as defensins and lysozyme.

A third mechanism by which elevated Cl^- efflux might augment pulmonary colonisation involves the effects of Cl^- concentration on neutrophils. CF lung disease is characterised by excessive neutrophilia and yet *P. aeruginosa* and *B. cepacia* flourish, despite exaggerated activation of these immune cells. The molecular basis of this caveat has not been elucidated fully. An enlightening study, carried out by Tager *et al. (340)*, found that PMN exposed to elevated concentrations of Cl^- were impaired in their ability to kill *P. aeruginosa*, and these immune cells displayed enhanced apoptosis and lysis. Therefore, bacterial elevation of Cl^- efflux from airway epithelial cells could disarm and kill infiltrating neutrophils. Furthermore, this same investigation showed that release of the neutrophil chemoattractant IL-8 was potentiated by an abnormally high Cl^- concentration in the extracellular fluid. This observation also has important implications for CF.

In conclusion, the data presented here demonstrate clearly that both *P. aeruginosa* and *B. cepacia* activated a transepithelial Cl^- current in tracheal epithelial cells *in vitro*. However, the questions regarding which Cl^- channels are activated by each pathogen, and whether any differences may be linked with the functional expression of CFTR, remain to be answered completely. Nevertheless, these observations are of great interest and potential significance to CF.

CHAPTER 5:

Cytokine production by airway epithelial cells

5.1 Introduction.

Destruction to the pulmonary tissue in CF is mediated largely by the host and the situation is exacerbated by repeated and chronic bacterial infection. Much of this inflammation is the result of an abnormally high influx of neutrophils, which release cytotoxic metabolites into the extracellular milieu with devastating effects on the respiratory epithelium. The most potent chemoattractant for neutrophils is IL-8 *(121)*, which is released by monocytic cells and epithelial cells alike, particularly in response to the pro-inflammatory cytokines IL-1β and TNF-α *(341)*. The anti-inflammatory cytokine IL-10 is produced in the airways by T lymphocytes to switch off the inflammatory response once the infection is resolved *(342-344)*. A recent study *(109)* indicated that IL-10 might also be produced by bronchial epithelial cells, although the capacity of tracheal epithelial cells to secrete IL-10 has not been examined. Several *in vitro* studies have shown that CF-derived primary and transformed epithelial cell lines express elevated levels of IL-8 mRNA and protein compared with non-CF tissue *(50;52;345;346)*, suggesting that IL-8 makes a central and significant contribution to the early inflammatory environment within the CF lung. In addition, there is evidence that the clinically important bacteria *P. aeruginosa* and *B. cepacia* intensify this situation further by up-regulating IL-8 production in the lung *(117;347-350)*. Furthermore, it has been suggested that pulmonary levels of IL-10 are reduced in CF compared with the normal lung *(108-110)*. The aim of this section of work was to investigate the differential effects on IL-8 and IL-10 production in CF- versus non-CF epithelial cells in response to bacterial and pharmacological stimuli, using ELISAs specific for IL-8 and IL-10 proteins.

5.2 Analysis of Epithelial Cell Supernatants Using an ELISA Specific for IL-8 or IL-10.

Epithelial cells were grown in 12-well plates and stimulated in serum-free conditions with bacterial or pharmacological agonists. Supernatant from unstimulated epithelial cells was analysed to investigate any phenotype-specific differences in IL-8 or IL-10 production by CF- and normal epithelial cells before and after stimulation. Epithelial cells were stimulated with the proinflammatory cytokines IL-1β or TNF-α, or with 10-fold serial dilutions of bacterial preparations that consisted of culture supernatant only (S), bacterial cells only (C), or whole bacterial suspensions (W). LPS from *P. aeruginosa* was also tested in this assay at a range of different concentrations, since bacterial endotoxin has been shown to modulate many immune responses *(64;349;351)*.

5.3 Basal Production of IL-8 by CF- and Normal Epithelial Cells in Culture.

Figure 5.1 shows the relative levels of IL-8 that each of the four cell lines produces in the absence of stimulation, or when treated for 24 h with vehicle controls. It can be seen that CFTE cells secreted greater basal levels of IL-8 than normal HTE cells (ANOVA $p<0.05$). In contrast, there was no significant difference between IL-8 production from normal and CF phenotype pCEP cells in the absence of agonist stimulation (ANOVA $p>0.05$). Vehicle controls were assessed for their effect on each of the four cell lines, with respect to IL-8 production. No significant difference was found compared with unstimulated cells (ANOVA, $p>0.05$ in each cell line).

5.4 IL-8 Production by Proinflammatory Cytokine-Stimulated Epithelial Cells.

Both CF- and normal airway epithelial cell lines secreted elevated levels of IL-8 in response to IL-1β treatment in a dose-dependent manner (**Figure 5.2**). The most productive IL-1β concentration in each case was 10 ng/ ml, which induced a mean IL-8 concentration of approximately 30 ng/ 10^6 cells in HTE and pCEP-RF cultures, and 20 ng/ 10^6 cells in pCEP-2 cells, although less than 8 ng IL-8/ 10^6 cells was released into CFTE culture supernatant. IL-1β concentrations as low as 0.3 ng/ ml elevated IL-8 secretion from HTE and pCEP cells, although significant stimulation was observed only at a concentration of 3 ng/ ml in non-CF cells (Dunnett's test $p<0.01$), and at 10 ng/ ml IL-1β in pCEP-RF cells (Dunnett's test $p<0.05$). A minimum IL-1β concentration of 3 ng/ ml was required to cause a significant increase in chemokine secretion from CFTE cells ($p<0.01$), although the maximum IL-8 production was less than a third of that observed in HTE- or pCEP epithelial supernatants.

Similarly, a dose response curve of IL-8 secretion was constructed for cells stimulated with TNF-α and the results are shown in **Figure 5.3**. These histograms demonstrate that IL-8 was secreted in a dose dependent manner in TNF α–induced cells and that in three of the cell lines (HTE, CFTE and pCEP-RF), 30 ng/ ml TNF-α was the optimum stimulatory concentration. In pCEP-2 cells 100 ng/ ml TNF-α elicited further IL-8 secretion. As with IL-1β stimulation, the strongest responses to TNF-α were seen in pCEP-RF cells, which produced 15 times more IL-8 than unstimulated controls; and in HTE

cells, which upregulated IL-8 production 14-fold in the presence of 30 ng/ ml TNF-α. Non-CF phenotype pCEP-2 epithelial cells displayed more than 10-fold upregulation of IL-8 secretion. However, CFTE cells secreted only three times more chemokine than controls. Since the most efficient induction of IL-8 was observed with 10 ng/ ml of IL-1β and 30 ng/ ml TNF-α in most of the cell lines, these optimum concentrations were selected for subsequent proinflammatory cytokine-stimulation of respiratory epithelial cell lines.

The kinetics of IL-8 production by CF and normal epithelial cells in response to the proinflammatory cytokines IL-1β and TNF-α were investigated and the results are displayed in **Figure 5.4**. IL-8 secretion from CFTE and HTE cells followed similar kinetics over the first 8 h after treatment with both IL-1β and TNF-α (**Figure 5.4 A**). Initially secretion was low but within 3 h, the rate of IL-8 production began to rise until approximately 8 h after stimulation. CFTE cells produced IL-8 in response to IL-1β for 8-12 h only, but TNF induced IL-8 from CFTE cells twice within 24 h, first at 3 h and then at 12-18 h post stimulation. HTE cells responded in a similar manner throughout the duration of the time course to both TNF and IL-1β stimulation. A second phase of IL-8 production was induced approximately 18 h after HTE treatment with proinflammatory cytokines.

Biphasic IL-8 production after IL-1β and TNF-α stimulation was also seen in pCEP epithelial cells. Both cytokines induced IL-8 within 3 h. The second wave of IL-8 production came approximately 18 h after cytokine stimulation, although this effect was more rapid and stronger in pCEP-RF cells treated with

IL-1β (12 h post stimulation), as shown in **Figure 5.4 B.** In common with HTE and CFTE cells, the first IL-8 secretion phase lasted until 8-12 h after initial stimulation, whilst the second wave of chemokine production continued to rise 24 h after the start of the time course.

5.5 Effect of *P. aeruginosa* on airway epithelial cell IL-8 secretion.

Two invasive laboratory strains of *P. aeruginosa* were used to stimulate the epithelial cell lines. **Figure 5.5** shows that strain PAO1 did not augment IL-8 levels in the culture supernatant of normal or CF epithelial cells after stimulation for 24 h. In fact, PAO1-treated respiratory cells produced less IL-8 than the controls and this reduction in chemokine synthesis was statistically significant in CFTE cells (Dunnett's test $p<0.05$) that had been stimulated with whole-bacterial suspension (moi = 200).

In contrast, PAK had a more pronounced effect on the IL-8 production from airway epithelial cells (**Figure 5.6**). Compared with PAO1-treated cells and unstimulated controls, CFTE cells ($p<0.01$), HTE cells ($p<0.05$), and pCEP-2 cells ($p<0.05$) responded only to PAK supernatant, whilst pCEP-RF cells responded to both bacteria and supernatant from PAK cultures either alone or in combination ($p<0.01$).

It can be seen from **Figure 5.7** that *P. aeruginosa* LPS had no significant effect on the degree of IL-8 secretion by CFTE cells ($p>0.9$) HTE cells ($p>0.9$) or

pCEP-2 cells ($p>0.2$) compared with controls, although pCEP-RF cells responded to *P. aeruginosa* LPS at 300 ng/ ml (Dunnett's test, $p<0.05$).

5.6 The *P. aeruginosa* type III secretion system does not mediate epithelial IL-8 production.

ADP-ribosylating toxins such as exoS, exoT and exoU are secreted by *P. aeruginosa* via type III secretion, which requires contact between cells of the host and the infecting pathogen. Whole cell cultures of PAK wild-type and PAK mutants lacking either exoS (PAK 6) or exoT (PAK 5) or both toxins (PAK 3 and PAK 4) and a mutant lacking the type III secretion system global regulator gene *exsA* (PAK 2) were used to stimulate the airway epithelial cell cultures at a high infective ratio (moi = 200). IL-8 production more than doubled in HTE cells to a maximum of 4.5 ng/ 10^6 cells (PAK 3 stimulation); and increased 3-fold in pCEP-2 cells to a maximum of almost 5 ng/ 10^6 cells following stimulation with PAK 4. In pCEP-RF cells the IL-8 response was up-regulated almost 7-fold to approximately 7.5 ng IL-8/ 10^6 cells ($p<0.001$; ANOVA). In contrast, CFTE cells did not secrete any more IL-8 than control populations (**Figure 5.8**). Mutants lacking exoS or exoT were not impaired in their abilities to stimulate epithelial cell IL-8 production and no difference was detected in any cell line after treatment with a type III secretion system-deficient *P. aeruginosa* mutant (PAK 2) compared with WT PAK-induced chemokine production. Some slight differences in IL-8 induction were observed amongst PAK mutants, although none was significant ($p>0.05$; ANOVA). These effects were similar in all cell lines tested.

When bacterial cells and supernatant were tested separately for chemokine-inducing potential, three of the cell lines showed similar and significant responses to both fractions (**Figure 5.9 and Figure 5.10**). Interestingly, CFTE cells secreted as much as 4.8 ng IL-8/ 10^6 cells, which equates to more than twice as much IL-8 secretion in response to PAK supernatant stimulation as in control cells ($p<0.05$; ANOVA), but neither whole cell culture nor PAK cells alone had any effect in this cell line (**Figure 5.8 A, Figure 5.9 A and Figure 5.10 A**). In the presence of bacterial supernatant, IL-8 production increased in HTE by as much as 4-fold to approximately 8.5 ng IL-8/ 10^6 cells ($p<0.01$; ANOVA); in pCEP-2 cells approximately 2-fold to more than 3.4 ng/ 10^6 cells ($p<0.05$; ANOVA); and in pCEP-RF cells to almost 4.7 ng/ 10^6 cells, which was more than four times greater than unstimulated controls ($p<0.0001$; ANOVA). However, none of the PAK mutations affected the IL-8 inducing potency of either supernatant or bacterial cells in any of the cell lines ($p>0.05$; ANOVA).

5.7 IL-8 response to *B. cepacia* by respiratory epithelial cells.

Ten-fold serial dilutions were prepared of a genomovar III isolate of *B. cepacia*, strain J2315, and incubated with epithelial cells for 24 h. Resultant IL-8 concentrations in epithelial supernatants are shown in **Figure 5.11**. *B. cepacia* induced significant IL-8 secretion by HTE and both pCEP cell lines (ANOVA $p<0.001$), but failed to elevate IL-8 production in CFTE cells. At a high bacterial cell to epithelial cell ratio (moi = 200), HTE-derived epithelial cells responded more strongly to a combination of *B. cepacia* supernatant and cells together than to either fraction alone, although at a lower ratio (moi = 2), both cellular and supernatant *B. cepacia* fractions increased HTE IL-8 production in

comparison with resting cells. Compared with unstimulated controls, epithelial cells treated with *B. cepacia* produced up to 8-fold (pCEP-2) or 14-fold (HTE, pCEP-RF) more IL-8 (Dunnett's test, $p<0.01$). This trend was repeated at a 10-fold lower bacterial dilution (moi = 20) although less IL-8 was produced than at the higher infection ratio. In normal and CF-phenotype pCEP epithelial cells, *B. cepacia* cells alone or supernatant alone each induced IL-8 secretion, but only some of these effects were significant (**Figure 5.11**).

5.8 Summary of IL-8 induction from airway epithelial cells.

The responses of airway epithelial cells towards bacterial and pharmacological agonists are compared in **Figure 5.12.** A remarkable similarity can be seen between the responses of HTE cells towards IL-1β and *B. cepacia*, which both elevated IL-8 production by approximately 15-fold. CFTE cells also responded in a similar manner to these agonists in that neither induced IL-8 from this cell line. *B. cepacia* also mimicked the potent effect of IL-1β in pCEP-RF cells, whilst pCEP-2 cells produced slightly more IL-8 after stimulation with cytokine treatment than after stimulation with bacteria. It is also of interest to note that whilst *P. aeruginosa* supernatant alone was a potent inducer of epithelial cell IL-8, cellular and supernatant factors from cultures of *B. cepacia* were required in combination to increase epithelial IL-8 production maximally. Furthermore, the relative effects of *P. aeruginosa* and *B. cepacia* differed amongst the epithelial cell lines. CFTE cells responded only to *P. aeruginosa*, whilst HTE cells responded only half as strongly to *P. aeruginosa* as to *B. cepacia.* pCEP-RF cells produced more IL-8 than pCEP-2 cells in response to *P. aeruginosa*

supernatant, and this difference was augmented in cells stimulated with *B. cepacia.*

5.9 IL-10 could not be detected in supernatant from CF- or normal epithelial cells in culture.

It was found that coating the 96-well plates overnight with 4 μg/ ml anti-human IL-10 monoclonal antibody gave a stronger signal than a concentration of only 2 μg/ ml in the presence of a range of concentrations of human recombinant IL-10, so the higher concentration of coating antibody was utilised. Since an equally strong signal was obtained on plates coated with 50 μl/ well or 100 μl/ well, the smaller volume was selected. Finally, plates were incubated at room temperature, rather than at 37°C, since this procedure gave the stronger responses over a wide range of concentrations of primary and secondary anti-IL-10 antibodies (data not shown). However, no IL-10 was detected in supernatant from any of the epithelial cell lines used in this work, regardless of phenotype with respect to CFTR (data not shown).

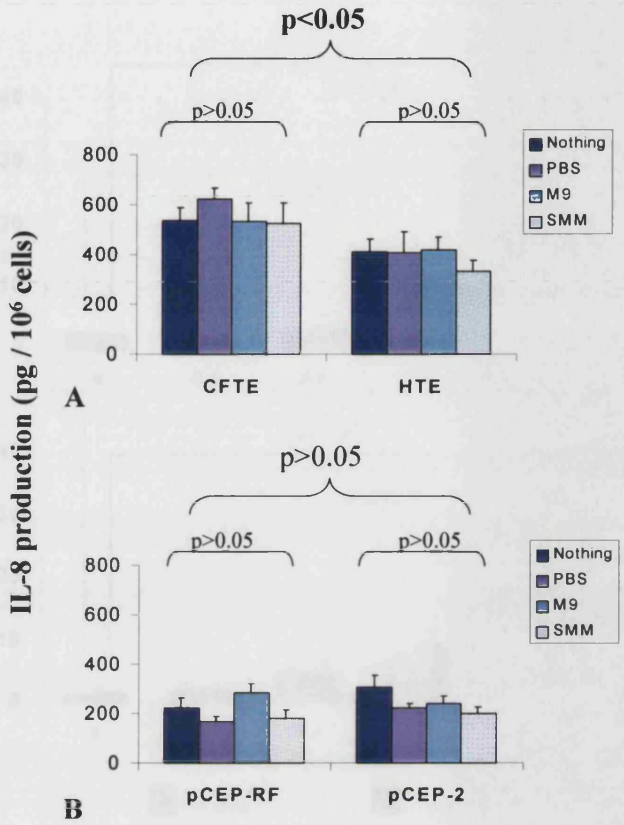


Figure 5.1 IL-8 production by unstimulated airway epithelial cells and 24 h after treatment with vehicle controls. Statistical analysis was performed using ANOVA to compare vehicle controls with unstimulated cells for IL-8 production. Comparison was also made between CFTE cells and HTE cells *(A)*, and between pCEP-RF cells and pCEP-2 cells *(B)*. $p<0.05$ was considered significant, n=26.

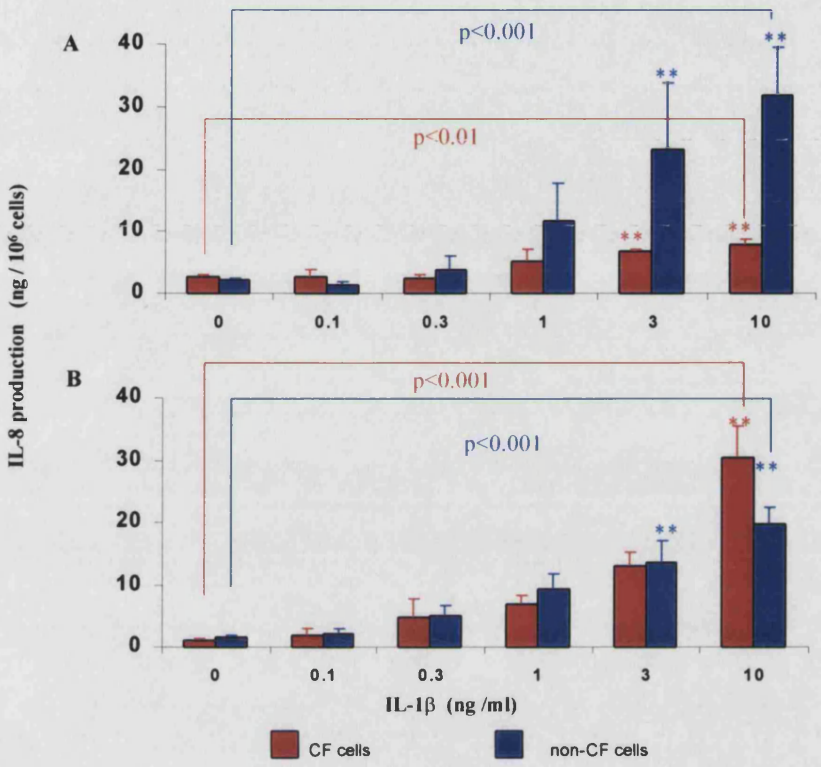


Figure 5.2 IL-8 production by CF- and non-CF- airway epithelial cells after 24 h stimulation with IL-1β *in vitro*. ***A***: CFTE and HTE ; ***B***: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA to compare data with untreated controls (0 ng/ml), followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant. (* $p<0.05$; ** $p<0.01$). "0 ng/ml" n=26; "10 ng/ml" n=11; all others n=3.

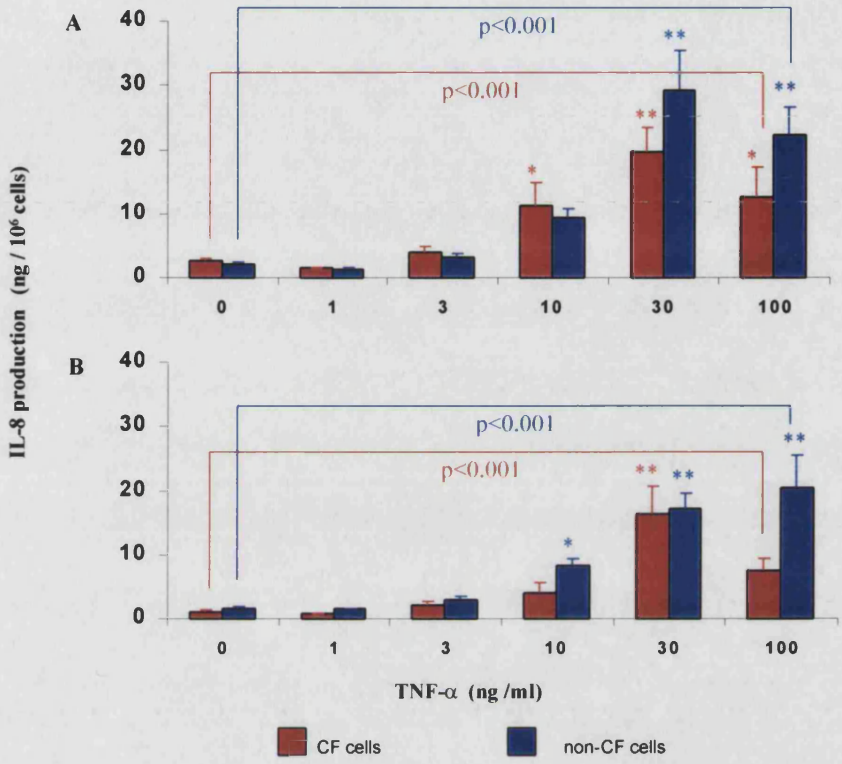


Figure 5.3 IL-8 production by CF- and non-CF- airway epithelial cells after 24 h stimulation with TNF-α *in vitro*. ***A***: CFTE and HTE ; ***B***: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA to compare stimulated cells with untreated controls (0 ng/ml), followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant. (* $p<0.05$; ** $p<0.01$). "0 ng/ml" n=26; "30 ng/ml" n=10; all others n=3.

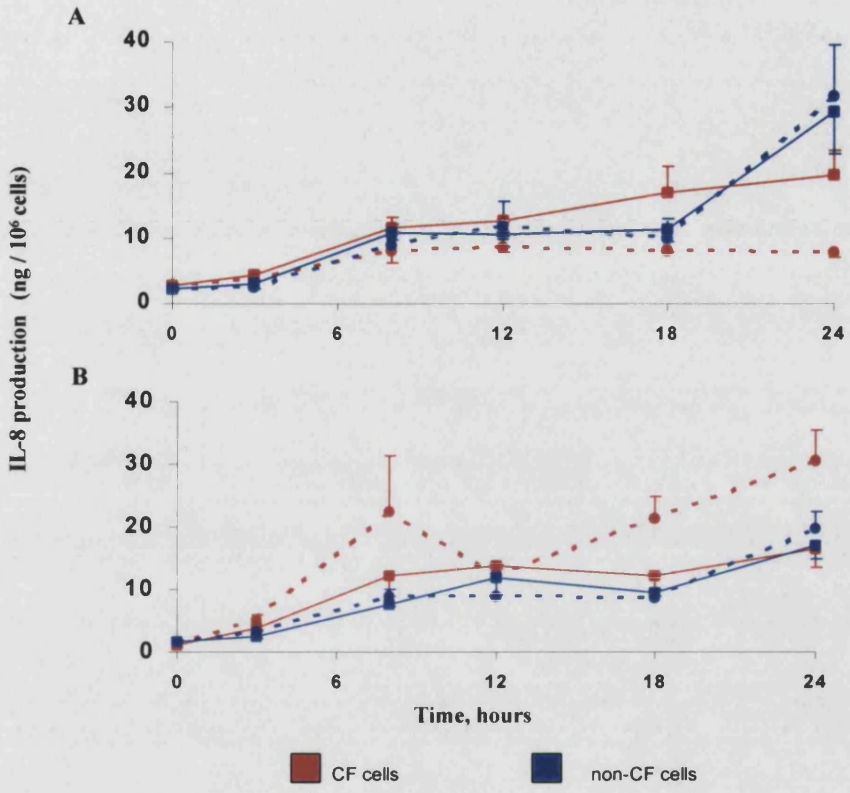


Figure 5.4 Kinetics of IL-8 production in airway epithelial cells after 24 h in response to pro-inflammatory cytokines *in vitro* (mean +/- SEM). *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. Circle with dotted line, IL-1β; square with solid line, TNF-α. t=0h, n=26; t=6h-18h n=3; t=24h n=26.

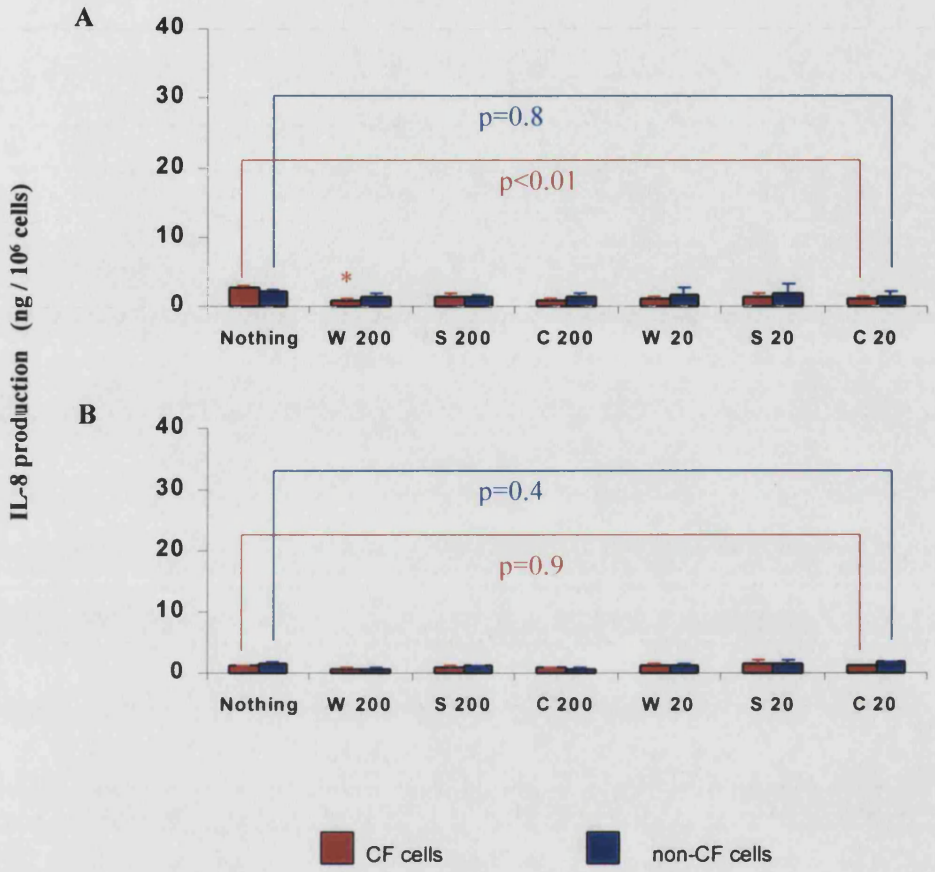


Figure 5.5 Effect of stimulation with *P. aeruginosa* PAO1 for 24 h on IL-8 production by airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. [W: whole bacterial suspension; S: cell-free bacterial culture supernatant; C: supernatant-free bacterial cells. Number indicates ratio of bacterial to epithelial cells]. Statistical analysis was performed using ANOVA to compare stimulated cells with untreated controls, followed by Dunnett's test for multiple comparisons where $p<0.05$ (*) was considered significant. Mean values + SEM are shown. "Nothing" n=26; all others n=3.

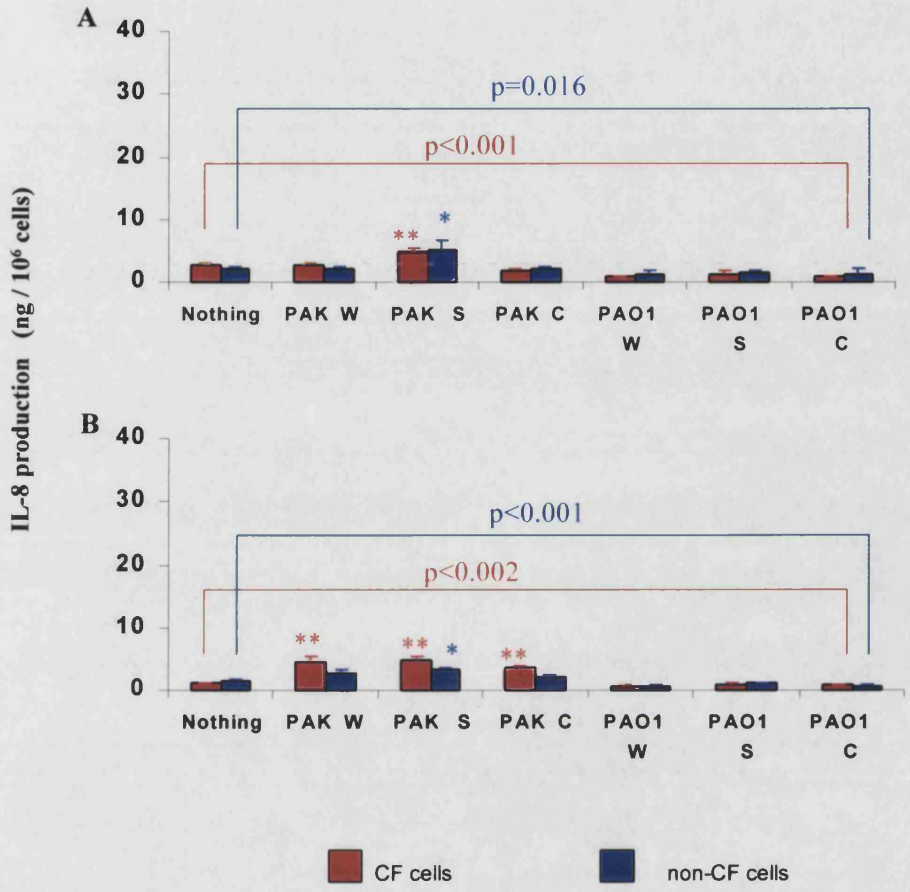


Figure 5.6 Comparison of the effects of stimulation with *P. aeruginosa* strains PAO1 and PAK for 24 h on the production of IL-8 from airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. [W: whole bacterial suspension; S: cell-free bacterial culture supernatant; C: supernatant-free bacterial cells. Number indicates ratio of bacterial to epithelial cells]. Statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test, comparing stimulated cells with untreated controls, where $p<0.05$ was considered significant, (* $p<0.05$; ** $p<0.01$). Mean values + SEM are shown. "Nothing" n=26; all others n=3.

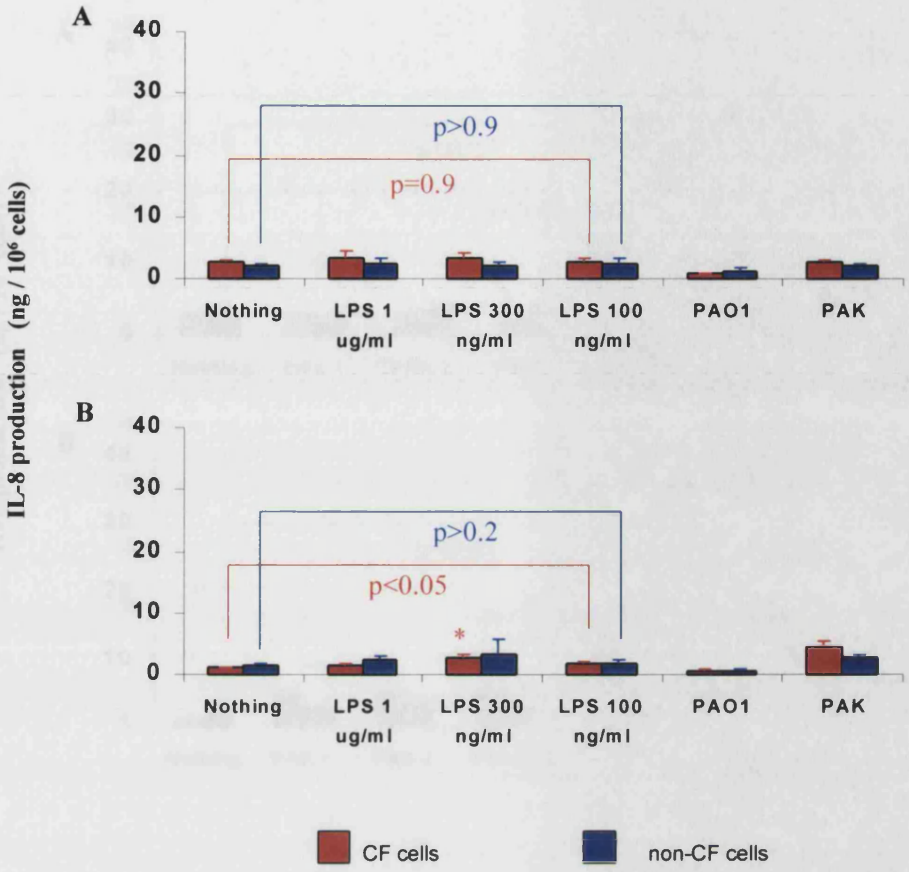


Figure 5.7 Comparison of effects of stimulation with *P. aeruginosa* LPS and strains PAO1 and PAK for 24 h on the production of IL-8 from airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test, comparing stimulated cells with untreated controls, where $p<0.05$ was considered significant. (* $p<0.05$). Mean values + SEM are shown. "Nothing" n=26; all others n=3.

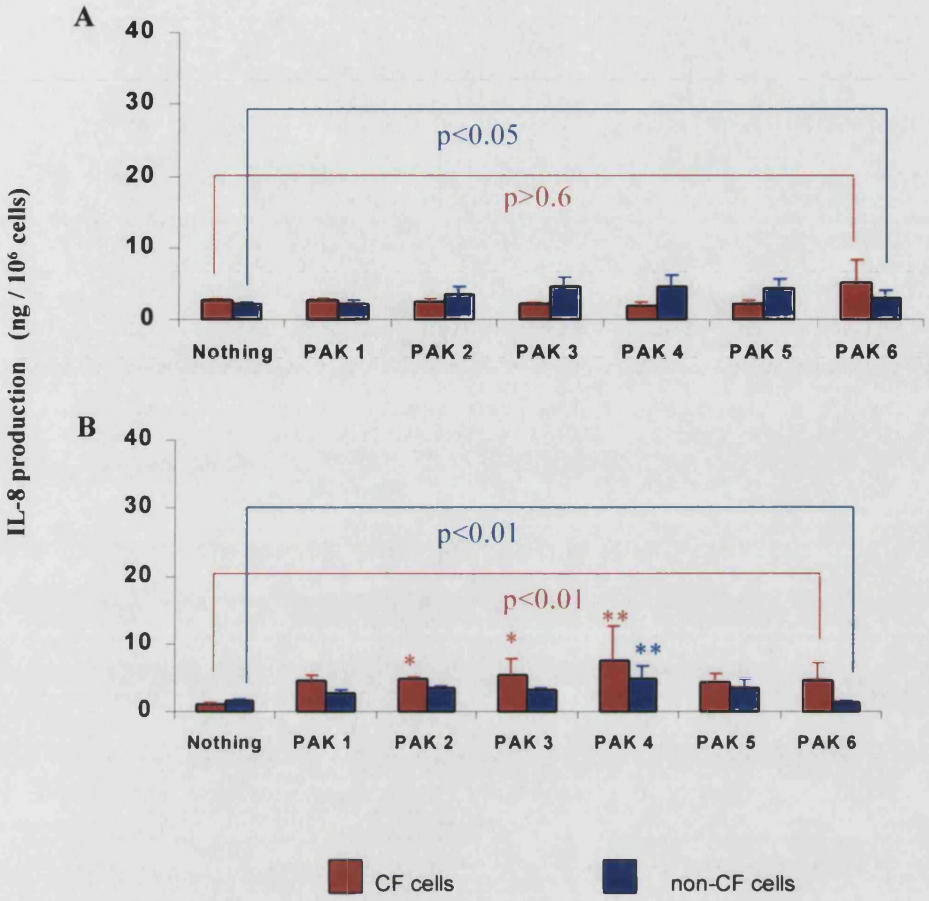


Figure 5.8 Comparison of the effects of stimulation with whole bacterial cell suspensions (W) of *P. aeruginosa* PAK mutants for 24 h on the production of IL-8 from airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test, comparing stimulated cells with untreated controls, where $p<0.05$ was considered significant. (* $p<0.05$; ** $p<0.01$). Mean values + SEM are shown. "Nothing" n=26; all others n=3. No significant difference was detected between mutants, ANOVA $p>0.05$ for each cell line.

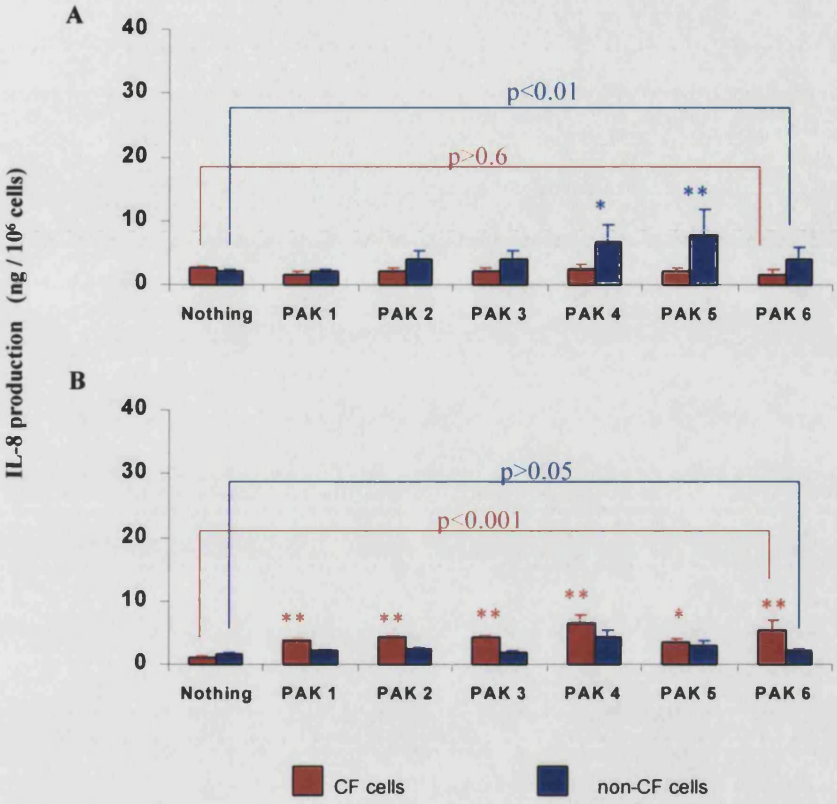


Figure 5.9 Comparison of the effects of stimulation with *P. aeruginosa* PAK mutant bacterial cells (C) for 24 h on the production of IL-8 from airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test, comparing stimulated cells with untreated controls, where $p<0.05$ was considered significant. (* $p<0.05$; ** $p<0.01$). Mean values + SEM are shown. "Nothing" n=26; all others n=3. No significant difference was detected between mutants, ANOVA $p>0.05$ for each cell line.

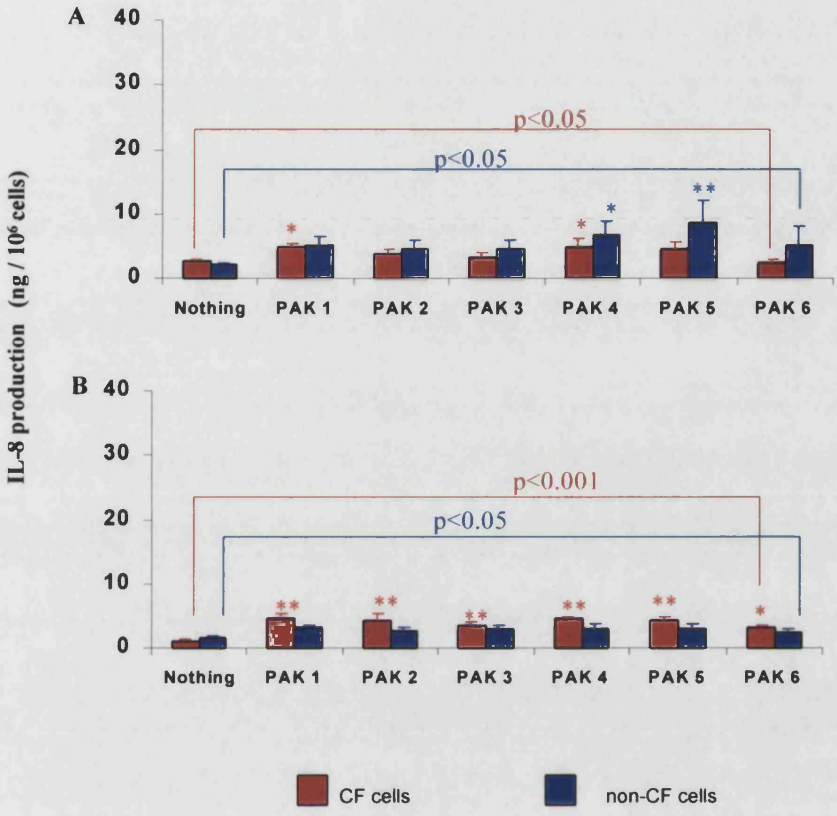


Figure 5.10 Comparison of the effects of stimulation with *P. aeruginosa* PAK mutant bacterial supernatant (S) for 24 h on the production of IL-8 from airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. Statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test, comparing stimulated cells with untreated controls, where $p<0.05$ was considered significant. (* $p<0.05$; ** $p<0.01$). Mean values + SEM are shown. "Nothing" n=26; all others n=3. No significant difference was detected between mutants, ANOVA $p>0.05$ for each cell line.

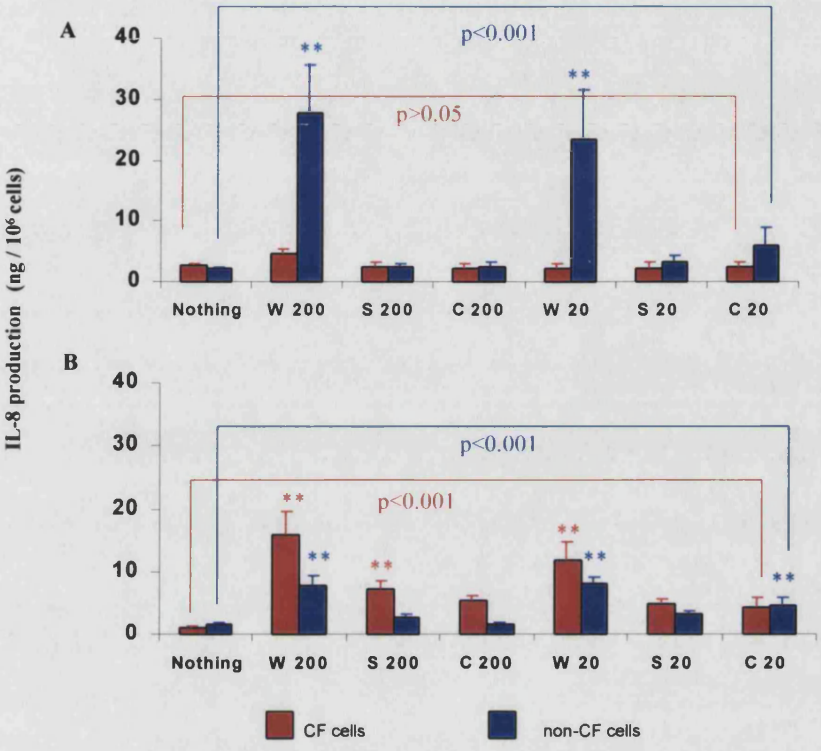


Figure 5.11 Effect of stimulation with *B. cepacia* J2315 for 24 h on IL-8 production by airway epithelial cells *in vitro*. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. [W: whole bacterial suspension; S: cell-free bacterial culture supernatant; C: supernatant-free bacterial cells. Number indicates ratio of bacterial to epithelial cells]. Statistical analysis was performed using ANOVA to compare stimulated cells with untreated controls, followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant (* $p<0.05$; **$p<0.01$). Mean values + SEM are shown. "Nothing" n=26; all others n=3.

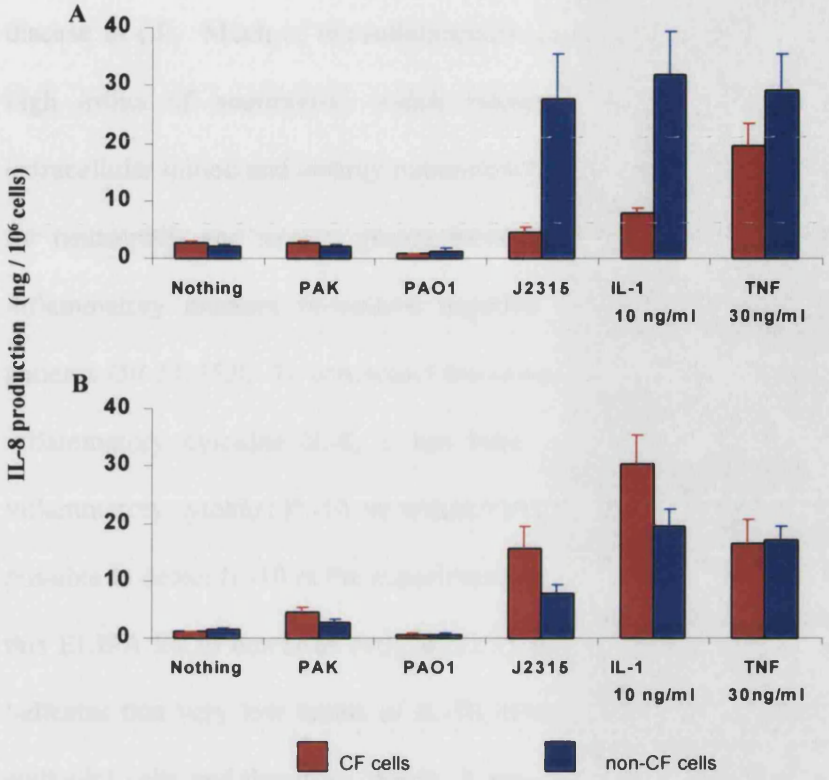


Figure 5.12 Comparison of IL-8 production *in vitro* by airway epithelial cells following stimulation for 24 h with bacterial and pharmacological agonists. *A*: CFTE and HTE; *B*: pCEP-RF and pCEP-2. (* $p<0.05$; **$p<0.01$). Statistical analysis was performed using ANOVA to compare stimulated cells with untreated controls, followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant (* $p<0.05$; **$p<0.01$). Mean values + SEM are shown. "Nothing" n=26; IL-1β n= all others n=3.

5.10 Discussion.

It is now accepted widely that an ineffective and exaggerated inflammatory response as a result of mutations in the CFTR gene underscores chronic lung disease in CF. Much of the inflammation can be attributed to an abnormally high influx of neutrophils, which release cytotoxic metabolites into the extracellular milieu and destroy pulmonary tissue. IL-8 is strongly chemotactic for neutrophils and several groups have reported elevated concentrations of inflammatory markers in culture negative airway tissue derived from CF patients *(50-53;352)*. To compound this abnormally high production of the pro-inflammatory cytokine IL-8, it has been reported that levels of the anti-inflammatory cytokine IL-10 are reduced in CF *(109)*. Unfortunately, it was not possible to detect IL-10 in the experiments in this study, despite the potential of this ELISA kit to detect as little as 31.25 pg IL-10/ ml. This negative result indicates that very low levels of IL-10, if any at all, are secreted by tracheal epithelial cells and therefore clearly, it was not possible to determine whether lack of functional expression of CFTR reduced the anti-inflammatory potential of this region of the lung. However, it would not be unreasonable to speculate that IL-10 may be more important in the distal rather than proximal airways, which could account for the negative result obtained in this study.

Data from the present study corroborated the idea that certain *cftr* mutations are associated with a hyper-inflammatory phenotype, showing that CFTE cells produced more IL-8 than HTE cells under unstimulated conditions (**Figure 5.1**). Unexpectedly, but of significant interest is that overexpression of the CFTR- R domain in the HTE-derived cell line pCEP-RF did not cause this effect, since

quiescent pCEP-RF and pCEP-2 cells did not secrete different levels of IL-8 (**Figure 5.1**). This finding is consistent with a recent report by Kube *et al. (353)*, which demonstrated that unstimulated pCEP-2 and pCEP-RF cells produce similar concentrations of IL-8. Therefore, it is likely that a physiological function of CFTR other than its chloride channel activity is responsible for the excessive inflammatory phenotype of the CF lung. However, some differences were detected between CF and normal pCEP cells after stimulation.

The inflammatory phenotype in naïve respiratory CF tissue is exacerbated by repeated and chronic infection with bacteria, viruses and fungi, which activate the immune systems further. Localised activated macrophages and the airway epithelium secrete both IL-1β and TNF-α into the airway lumen activating the respiratory epithelium in a time- and dose-dependent manner, which was reflected in the current studies with these epithelial cells (**Figure 5.2, Figure 5.3 & Figure 5.4**). IL-1β caused dramatic elevation in IL-8 production by HTE, pCEP-2 and pCEP-RF cells but had little effect in CFTE cells (**Figure 5.2**); whilst TNF-α enhanced production of this chemokine in each of the four cell lines (**Figure 5.3**). Cytokine-induced IL-8 secretion occurred at a similar rate in both HTE and CFTE cell lines within the first 12 h of stimulation, but IL-1β-induced IL-8 secretion ceased after this time (**Figure 5.4**). In contrast, a second elevation in IL-8 production began approximately 18 h after initial stimulation in pCEP-RF cells treated with TNF-α and in both non-CF cell lines treated with TNF-α and IL-1β. Discrepancies between the responses towards the two cytokines may be the result of variable degrees of receptor expression or

differences in the activities of downstream signalling cascades in each cell line. In common with the work presented here, studies by Palfreyman *et al. (117)* and Chen *et al. (354)* demonstrated biphasic IL-8 mRNA production in the respiratory epithelial cell line A549. Increased mRNA synthesis could explain reasonably the biphasic IL-8 protein secretion displayed in **Figure 5.4** in this study and may reflect two different types of IL-8 regulation, at the transcriptional and post-transcriptional levels.

Several studies have demonstrated that the concentrations of both IL-1β and TNF-α are increased in the BALF of CF patients *(103;173;355)*, which could enhance neutrophil influx via epithelial cell stimulation. Epithelial cells are not the only source of IL-8 in the lung since serous gland cells, macrophages and neutrophils themselves are rich producers of this chemokine at rest and following cytokine or bacterial activation *(345;356-359)*. Regardless of whether this elevation of IL-8 is the result of respiratory infection or a consequence of the underlying *cftr* mutation, it is clear that increased proinflammatory cytokine release into the pulmonary environment will have important ramifications for chemokine secretion, precipitating the infiltration of neutrophils into the CF lung via autocrine and paracrine IL-8.

Examination of the effects of the CF pathogens *P. aeruginosa* and *B. cepacia* revealed some important differences between the patterns and modes of IL-8 induction in the lung by these bacteria. Two different isolates of *P. aeruginosa* were employed, namely PAO1 and PAK, and both are non-invasive laboratory strains. Unexpectedly, even when there were 200-fold more bacterial cells than

epithelial cells, PAO1 did not induce IL-8 production and in fact, there was reduced IL-8 secretion by CFTE cells at this infective ratio. One possible explanation for this observation is epithelial cells were being killed by PAO1 at this multiplicity of infection. It has been reported widely that certain strains of *P. aeruginosa* may precipitate epithelial cell death *(312;360-363)* and clearly, this would result in reduced IL-8 output over 24 h. PAO1 toxicity towards the respiratory epithelial cells was not measured, although gross microscopic examination of the monolayers after 24 h suggested that epithelial cell viability may have been compromised by PAO1 co-incubation (data not shown). Indeed, a recent study examining the effects of this strain of *P. aeruginosa* on epithelial IL-8 secretion co-incubated pCEP epithelial cells with PAO1 for only 60 min *(353)*, suggesting that epithelial cell viability may be reduced by prolonged exposure to this strain of *P. aeruginosa.* A brief exposure to PAO1 at infective ratios between 0.1 and 100 bacteria per epithelial cell, elicited significant IL-8 release from both pCEP-RF and pCEP-2 epithelial cells after 24 h *(353)*. The data presented here do not support these observations, but they were generated using a modified incubation strategy. Therefore, it is possible that whilst basal levels of IL-8 were still detected in pCEP cells, further IL-8 release was prevented because of toxic effects of PAO1. Alternatively, PAO1 may inhibit host intracellular signalling cascades that lie upstream of IL-8 production. This effect was not observed at lower infective ratios or in cells treated either with supernatant or with bacterial cells alone, so that inhibition of IL-8 production in CFTE cells by PAO1 required a synergistic combination of both secreted and structural bacterial factors. Only CFTE cells produced less IL-8 than controls after PAO1-treatment, so the inhibitory effects of PAO1 on IL-8 production

may relate to CFTR genotype. In contrast, *P. aeruginosa* PAK elevated IL-8 secretion from all four of the cell lines (**Figure 5.6**), suggesting either that the rate of IL-8 induction was greater or the rate of PAK-induced cell death was lower - if at all - than with PAO1 stimulation.

Many *P. aeruginosa* factors have been implicated in the generation of an inflammatory response in the lung via IL-8 secretion, including structural components such as flagella *(116)* and LPS *(349;364)*; and secreted products including nitrate reductase *(357)* autoinducers *(116)*, haemolytic phospholipase C *(195)*, pyocyanin *(203;365)*, 1-hydroxyphenazine *(365)* and alginate *(212)*. Furthermore, several studies describe the effects of *P. aeruginosa* products, such as exotoxin A, on IL-8 secretion by neutrophils and monocytic cells of the blood and airways *(212;359;366;367)*, and this secondary source of IL-8 could also contribute to pulmonary neutrophilia. The immunogenic role of *P. aeruginosa* LPS is controversial, however, since this and other work have shown that *P. aeruginosa* LPS does not elevate epithelial IL-8 *(359)*, although these differences may be related to the specific strains of *P. aeruginosa* from which the LPS was obtained. An interesting investigation by Ernst *et al. (351)* identified aminoarabinose and palmitate in the lipid A component of LPS from CF isolates of *P. aeruginosa*. This unusual composition conferred a resistance to defensins upon these bacterial strains and made the LPS more immunogenic than that from non-CF *P. aeruginosa* strains. Thus, whilst the LPS serotype tested here did not augment airway epithelial IL-8 production *in vitro*, the situation may be different in the CF lung *in vivo*.

In addition to these virulence factors, *P. aeruginosa* produces toxins such as exoS and exoT, both of which possess ADP-ribosylating activity that can interfere with host intracellular signalling cascades *(244;249;363;368)*. These enzymes are secreted directly into host cells via type III secretion system apparatus. It has been reported that *P. aeruginosa* exoS induces the secretion of proinflammatory mediators from peripheral blood mononuclear cells (PBMCs) *(369)* although no published data are available concerning the effects of exoS on epithelial IL-8 release. However, the results presented here lend strong support to the idea that *P. aeruginosa* exoS does not interfere with the regulated production of IL-8 by respiratory epithelial cells since PAK mutants lacking exoS (PAK 3, PAK 4 and PAK 5) did not differ in their abilities to induce IL-8 from epithelial cells, compared with the parental PAK strain. In the same way, PAK mutants lacking exoT (PAK 3, PAK 4 and PAK 6) were not affected in their potency to induce epithelial IL-8 (**Figure 5.8 & Figure 5.9**). This hypothesis was strengthened by the observation that a type III secretion system knockout (*exsA* mutant, PAK 2) elicited similar IL-8 to wild-type PAK, demonstrating that *P. aeruginosa* induced IL-8 production via a type III secretion system-independent mechanism.

Evidence for a type III secretion system-independent induction of IL-8 was reinforced by the results shown in **Figure 5.10** that bacterial-free supernatant from each of the PAK mutants elevated IL-8 production from all of the cell lines. Indeed, CFTE cells responded only to bacterial supernatant and IL-8 release was not elevated in the presence of PAK cells (compare **Figure 5.8 A, Figure 5.9 A** and **Figure 5.10 A**). Furthermore, there was no significant

difference amongst supernatants from mutant and wild-type PAK *P. aeruginosa* to elicit IL-8 from the respiratory epithelial cultures. Since the type III secretion system requires cell-to-cell contact, bacterial supernatant alone would not mediate type III secretion system effects.

It is interesting to note that no inhibition of IL-8 production was observed after CFTE stimulation with PAK cells at the same infective ratio as PAO1 (moi = 200). Since PAK mutants lacking exoS are defective in the induction of epithelial apoptosis *(312)*, any inhibitory effects on chemokine production that were related to epithelial cell death would almost certainly have been obvious in the cell lines treated with PAK 3, PAK 4 or PAK 5, compared with wild-type stimulated cells. No significant difference was observed between the potency of PAK 1 and exoS mutants, illustrating that epithelial cell apoptosis could not explain the lack of response of CFTE cells to *P. aeruginosa* PAK stimulation.

Immune cell death has been noted previously in the presence of *B. cepacia* *(280;370)* but the data presented in **Figure 5.11** suggest that this bacterial strain was not cytotoxic towards epithelial cell lines in this study. HTE and pCEP cell lines all responded significantly to *B. cepacia* stimulation, whilst CFTE cells produced slightly more IL-8 at a high infective ratio of *B. cepacia* (moi = 200) than unstimulated controls. Although this latter increase was not significant, it is unlikely that the unresponsiveness was the result of CFTE cell apoptosis. A synergistic effect of *B. cepacia* bacteria and supernatant was observed in non-CF epithelial cells and was particularly striking in HTE cells, implying the involvement of both secreted and structural *B. cepacia* factors. Similarly, CFTE

cells only increased IL-8 secretion after stimulation with a combination of bacteria and supernatant, and both fractions elevated IL-8 in pCEP-RF cells. Previous reports allude to the involvement of a heat-labile secreted *B. cepacia* factor in IL-8 generation in airway cells, whose identity was not determined but was not related to AHLs *(117)*; and to the possible role of *B. cepacia* LPS in IL-8 generation in the lung, via the release of TNF-α from neutrophils *(62)*. This latter possibility is especially intriguing given that the IL-8 response to stimulation with cytokines and *B. cepacia* was similar in three of the cell lines **(Figure 5.11)**. An alternative explanation might be that *B. cepacia* activates the same epithelial cell cytokine receptors or convergent signalling cascades. For example, the Toll-like receptor, which mediates endotoxin recognition in the lung *(371-373)*, shares many features with the IL-1 receptor *(374)*. The data presented here do not exclude a role for AHLs in IL-8 induction but other secreted factors such as siderophores, melanin or digestive enzymes may also be involved. In particular, haemolysin has been shown specifically to modulate the pulmonary immune response by inducing the degranulation of neutrophils *(280)*. Likely candidate structural components of *B. cepacia* that may activate epithelial chemokine production include LPS and adhesion proteins.

In conclusion, CFTE cells and pCEP-RF cells generally displayed the most extreme IL-8 responses to stimulation, although the former were weak and the latter were strong; and normal airway epithelial cells responded mostly at a level in between. In addition, the likelihood that a complex and synergistic interaction exists between bacteria and biochemical mediators of inflammation in the induction of IL-8 should not be discounted although this possibility was

not investigated. Differentiation between the CF and normal phenotypes with respect to IL-8 production is not straightforward, although it is clear that some differences do exist.

CHAPTER 6:

The role of MAP kinase cascades in airway epithelial cell IL-8 production

6.1 Introduction

It is clear from the previous chapter and from other investigations that IL-8 plays a pivotal role in the inflammatory process and tissue pathology of CF. However, the nature of the upstream signalling events that lead to the production of respiratory IL-8 are complex and remain incompletely characterised. Delineation of these cascades could identify targets for therapeutic intervention that might alleviate the host-derived symptoms associated with chronic lung disease, whilst maintaining protection against the spread of infection. This chapter aims to address this issue and describes a number of approaches used to investigate potential events in pulmonary signal transduction.

Several published observations suggest that IL-8 secretion may be mediated at least in part by the activation of NFκB or the activation of MAP kinase signalling cascades *(347;354;364;375-378)*. In order to determine the role of MAP kinases in normal and CF-respiratory IL-8 production, two strategies were adopted. Firstly, IL-8 production was measured by ELISA in epithelial monolayers stimulated with *B. cepacia* or with the pro-inflammatory cytokines IL-1β or TNF-α, in the presence of specific inhibitors of components of the MAP kinase pathway. In addition, the activation of ERK in response to bacterial stimuli was investigated by Western blotting using anti-sera specific to phosphorylated (active) ERK. Measurement of phosphorylated ERK is an indirect method of assaying ERK activity.

6.2 Effect of MAP Kinase Inhibitors on Airway Epithelial Cell IL-8 Production.

6.2.1 Reduced IL-8 production by airway epithelial cells following specific antagonism of p38 MAP kinase.

Epithelial cells were incubated for 1 h with SB-203580, a specific inhibitor of p38 MAP kinase *(379)*, and then stimulated with 10 ng/ ml IL-1β, 30 ng/ ml TNF-α, or *B. cepacia* J2315 (moi = 200) for 24 h. In the presence of SB-203580, IL-1β induced similar levels of IL-8 in CFTE cells to those detected in the supernatant from uninhibited CFTE cells (**Figure 6.1 A**). However, SB-203580 diminished IL-8 production significantly ($p<0.05$; ANOVA) in IL-1β-stimulated HTE cells compared with HTE cells treated with IL-1β alone (**Figure 6.1 A**). Maximal inhibition of IL-8 production in IL-1β-stimulated HTE cells was achieved with 10 μM SB-203580, which reduced the amount of IL-8 in epithelial supernatant by 70 % from 30.0 ng/ 10^6 cells to 7.0 ng/ 10^6 cells. This value corresponds to an 82 % reduction in induced IL-8. In addition, both 30 μM and 1 μM SB-203580 displayed potent inhibitory activities, each decreasing stimulated IL-8 production by approximately 63 % to 13.0 ng/ 10^6 cells, compared with IL-1β-stimulated cells alone. At this concentration of IL-1β, 3 μM SB-203580 had less effect on chemokine secretion.

Both pCEP-RF and pCEP-2 cells also produced less IL-8 in the presence of SB-203580 after stimulation with IL-1β and this effect was dose dependent (**Figure 6.1 B**). IL-1β induced 30.0 ng IL-8/ 10^6 cells in pCEP-RF cells but this was

reduced to 5.0 ng/ 10^6 cells by 10 μM SB-203580, equivalent to approximately 87 % inhibition. IL-8 secretion was also diminished by more than 50 % in the presence of either 30 μM or 3 μM SB-203580 in pCEP-RF cells. In the same way, both 30 μM and 10 μM SB-203580 decreased IL-8 production in pCEP-2 cells by approximately 70 % following stimulation with IL-1β. Lower concentrations (0.3 μM – 3 μM) of this p38 MAP kinase antagonist also displayed some inhibitory potential in both pCEP cell lines.

A stronger effect of SB-203580 pre-treatment on IL-8 production was seen in epithelial cell lines that had been stimulated with TNF-α. CFTE cells produced almost 20 ng IL-8/ 10^6 cells after stimulation with TNF-α, but this chemokine production was decreased by 98 % to 3.2 ng IL-8/ 10^6 cells in the presence of 30 μM SB-203580 (**Figure 6.2 A**). This residual IL-8 secretion was only slightly higher than basal production. Even 0.3 μM of this inhibitor diminished the TNF-α-induced secretion of IL-8 in CFTE cells. HTE cells also responded to the presence of SB-203580 in a dose dependent manner, as shown in **Figure 6.2 A**. IL-8 production was inhibited by 82 % in TNF-α-stimulated HTE cells that had been pre-treated with 30 μM SB-203580 and again, even 0.3 μM SB-203580 decreased production of the chemokine by 50 % in these cells. Antagonism of IL-8 secretion by this p38 MAP kinase inhibitor at each concentration was similar in both pCEP-RF and pCEP-2 cells, as shown in **Figure 6.2 B**. In these epithelial cells, the strongest inhibitory effect of SB-203580 was detected at 30 μM, causing a reduction of 78 % and 75 % in IL-8 secretion from pCEP-RF and pCEP-2 cells, respectively.

The slight increase in IL-8 production by CFTE cells following *B. cepacia* stimulation was antagonised by the p38 MAP kinase inhibitor, SB-203580 (**Figure 6.3 A**). Concentrations of both 30 μM and 10 μM caused a visible decrease in *B. cepacia*-induced IL-8 secretion to 3.0 ng/ 10^6 cells and 2.8 ng/ 10^6 cells, respectively. These values are still marginally higher than in quiescent CFTE cells, which generated 2.6 ng IL-8/ 10^6 cells but they correspond to the fact that SB-203580 abrogated more than 90 % of the IL-8 response to *B. cepacia* in CFTE cells. However, since *B. cepacia* did not increase CFTE IL-8 production visibly these findings may not be relevant and indeed, were not statistically significant (ANOVA, $p>0.05$).

A pronounced effect of p38 inhibition was seen also in HTE and pCEP cells. After bacterial stimulation, almost 28 ng IL-8/ 10^6 cells was detected in HTE supernatant but this value was reduced to just 4.6 ng IL-8/ 10^6 cells in the presence of 30 μM SB-203580 (90 % inhibition); and to 6.0 ng IL-8/ 10^6 cells with 10 μM inhibitor (**Figure 6.3 A**). Even 0.3 μM SB-203580 abrogated some of the bacterial-induced IL-8 production in these cells. The dose-dependent inhibition of IL-8 production in pCEP cell lines by SB-203580 was striking (**Figure 6.3 B**). Bacterial stimulation elevated chemokine synthesis from approximately 1.2 ng IL-8/ 10^6 cells in pCEP-RF cells and 1.6 ng IL-8/ 10^6 pCEP-2 cells to 15.8 ng/ 10^6 cells and 7.8 ng/ 10^6 cells, respectively. However, pre-incubation with 30 μM SB-203580 decreased these values to 1.4 ng IL-8/ 10^6 pCEP-RF cells and 0.6 ng IL-8/ 10^6 pCEP-2 cells. These values are lower than in unstimulated cells, corresponding to 100 % inhibition of bacterial-

induced chemokine in pCEP cells. IL-8 production increased gradually as the inhibitor concentration was reduced and once again, 0.3 μM antagonist was sufficient to prevent maximal induction of IL-8 in these cells. Fifty percent less IL-8 was formed in pCEP-RF cells that had been pre-treated with 0.3 μM SB-203580 then stimulated with *B. cepacia*, compared with stimulated control cells. Similarly, this concentration of inhibitor was sufficient to reduce pCEP-2 IL-8 secretion to 4.4 ng/ 10^6 cells, representing 54 % less chemokine induction **(Figure 6.3 B)**.

6.2.2 Reduced IL-8 production by airway epithelial cells following specific antagonism of ERK MAP kinase.

Epithelial cells were incubated for 1 h with PD-098059, a specific inhibitor of ERK MAP kinase *(142)*, and then stimulated with 10 ng/ml IL-1β, 30 ng/ml TNF-α or *B. cepacia* J2315 (moi = 200) for 24 h. IL-8 concentration in the epithelial supernatant was measured by ELISA and the results are displayed in **Figures 6.4-6.6**. Pre-treatment with PD-098059 caused a visible reduction in IL-1β-induced IL-8 production in both pCEP and HTE cells in a dose-dependent manner, and these effects were significant in pCEP cell lines ($p<0.05$; ANOVA). Both 10 μM ($p<0.05$ in pCEP-2; Dunnett's test) and 3 μM PD-098059 decreased IL-1β-induced chemokine production by at least 50 % in pCEP cells from 30.0 ng IL-8/ 10^6 cells (pCEP-RF) and 20.0 ng IL-8/ 10^6 cells (pCEP-2) as shown in **Figure 6.4 B.** A similar degree of inhibition was seen in HTE cells that had been pre-treated with 10 μM PD-098059 before stimulation with IL-1β **(Figure 6.4 A)**, in which IL-8 secretion was reduced from 32 ng/ 10^6 cells to 15 ng/ 10^6 cells. Essentially, IL-1β-induction of IL-8 in CFTE cells was

unaffected by the ERK inhibitor PD-098059 at these concentrations (**Figure 6.4 A**).

However, **Figure 6.5 A** shows that PD-098059 did reduce TNF-α-induced IL-8 secretion by CFTE cells significantly ($p<0.05$; ANOVA). The most potent inhibitory concentration of PD-098059 was 0.1 μM, which eliminated approximately 88 % of TNF-α-induced IL-8 production in CFTE cells and approximately 60 % inhibition of chemokine production was provided by higher concentrations of this compound. High (10 μM) levels of PD-098059 caused a clear reduction of TNF-α-induced chemokine secretion by HTE and pCEP-RF cells by 50 % and 67 %, respectively; and as little as 1 μM reduced IL-8 production following stimulation with TNF-α in the pCEP-RF and HTE cell lines (**Figure 6.5 A and B**). Significant inhibition of IL-8 production was seen in TNF-α-stimulated pCEP-2 cells ($p<0.05$; ANOVA) that had been pre-treated with the ERK inhibitor, diminishing chemokine production by 48 % from 18 ng/ 10^6 cells to less than 10 ng/ 10^6 cells in the presence of 10 μM or 1 μM PD-098959 (**Figure 6.5 B**).

B. cepacia-stimulated HTE cells secreted less than a third as much IL-8 in the presence of either 10 μM or 3 μM PD-098059 as did uninhibited controls (**Figure 6.6 A**). However, this apparently striking inhibition was not statistically significant ($p>0.3$; ANOVA). PD-098059 inhibited *B. cepacia*-induced IL-8 production by 71 % in CFTE cells to 3.2 ng IL-8/ 10^6 cells, compared with CFTE cells treated with *B. cepacia* J2315 alone and control cells (**Figure 6.6 A**). Both pCEP-2 and pCEP-RF cell lines secreted significantly

less IL-8 after stimulation with *B. cepacia* J2315 if they had been treated first with PD-098059, compared with uninhibited epithelial cells ($p<0.05$; ANOVA) as illustrated in **Figure 6.6 B** and this effect was significant at 10 μM PD-098059 in those cells expressing a normal phenotype. *B. cepacia* induced 16.0 ng IL-8/ 10^6 cells in pCEP-RF cells, which was reduced by approximately 75 % to less than 4 ng/ 10^6 cells in the presence of either 10 μM or 3 μM PD-098059. Bacterial-induced IL-8 was inhibited by more than 85 % in pCEP-2 cells, after pre-treatment with 10 μM PD-098059, to only 2.8 ng/ 10^6 cells. Similarly, 3 μM PD-098059 inhibited IL-8 production in pCEP-2 cells, reducing IL-8 induction by *B. cepacia* to less than 50 % of uninhibited levels.

6.2.3 Assessment of the toxicity of dicoumarol to airway epithelial cells.

Specific and selective inhibitors of extracellular regulated kinase (ERK) and p38 MAP kinase cascades are available commercially *(142;379)*, although an equivalent compound has not been reported for antagonising the *c*-Jun N-terminal kinase (JNK/SAPK) MAP kinase pathway. A recent study by Templeton *et al. (380)* described the potential of dicoumarol to fulfil this role. In their experiments, 150 μM dicoumarol reduced the activity of JNK by at least 90 %, whilst dose dependent inhibition was also displayed at lower dicoumarol concentrations *(380)*. However, dicoumarol also potentiated the induction of apoptosis by TNF-α *(380)* suggesting that dicoumarol is toxic to some mammalian cells.

To assess whether this coumarin derivative might be useful for examining the role of MAP kinase pathways in IL-8 production, dicoumarol toxicity towards

pCEP cells was investigated. To determine whether the epithelial cell lines were resistant to dicoumarol-induced cell death, pCEP-2 and pCEP-RF cells were grown in 6-well plates until almost confluent and incubated with dicoumarol for 24 h. Epithelial cell viability was assessed by trypan blue exclusion and the results are displayed in **Figure 6.7**.

After 3 h, only 85 % of pCEP-RF cells remained viable following treatment with 150 μM or 75 μM dicoumarol, respectively (**Figure 6.7 A and B**). Five hours later, only 60-70 % of the original pCEP-RF population had survived. After 12 h incubation, 25 % of cells had survived 150 μM dicoumarol, whilst 40 % of pCEP-RF cells retained an intact membrane in the presence of 75 μM dicoumarol at this time point. Similar trends were observed in pCEP-2 cells. Neither cell population survived a 24 h-incubation period with either 150 μM or 75 μM dicoumarol. **Figure 6.7 C** shows that even 38 μM dicoumarol killed 25 % of pCEP-RF and 30 % pCEP-2 cells after 8 h, and 50 % of pCEP-RF cells were dead within 12 h. pCEP-2 cells appeared slightly more recalcitrant to dicoumarol toxicity at this concentration however, since more than 65 % of the pCEP-2 population survived 12 h incubation with 38 μM dicoumarol. In contrast to samples treated with higher concentrations of dicoumarol, 20 % of each cell population remained viable in the presence of 38 μM dicoumarol after 24 h. Dicoumarol vehicle control (MilliQ adjusted to pH 9.8) had no effect on epithelial cell viability (**Figure 6.7 D**). Clearly, these data suggest that it would be difficult to distinguish between the toxic effects of dicoumarol and any inhibitory effects on MAP kinase signalling. Therefore, the potential of an alternative incubation strategy was assessed.

Epithelial cells were grown as before and then treated with dicoumarol for only 30 min before washing away excess dicoumarol and replacing with dicoumarol-free medium. After 24 h, epithelial cell viability was determined by trypan blue exclusion and the results are shown in **Figure 6.8.** A striking increase in viability was observed in the pCEP-2 cell line following only 30 min treatment with either 150 μM or 75 μM dicoumarol **(Figure 6.8 A and B)**, compared with cells that had been treated for 24 h with the inhibitor. At no time during the 24 h period did pCEP-2 viability drop below 75 % if cells had been incubated with dicoumarol for only 30 min, regardless of the concentration used. Although pCEP-RF cells did not resist the toxic effects of a 30 min exposure to 150 μM dicoumarol to the same extent as pCEP-2 cells, at least 50 % viability was observed in the pCEP-RF population even after 24 h. Furthermore, at least 75 % of the pCEP-RF population survived a 30 min exposure to 75 μM dicoumarol **(Figure 6.8 B)**. In view of these data, cells were exposed to dicoumarol for only 30 min in order to assess its potential as an inhibitor of IL-8 production in stimulated epithelial cells.

6.2.4 Reduced IL-8 production by airway epithelial cells following pre-treatment with dicoumarol.

Both CF and normal epithelial cells were incubated with dicoumarol for 30 min. Unabsorbed dicoumarol was washed away and replaced with dicoumarol-free medium containing IL-1β, TNF-α or *B. cepacia* J2315 as described previously. **Figures 6.9-6.11** show that IL-8 production was reduced considerably and in a dose-dependent manner by dicoumarol. IL-1β elicited 30.4 ng IL-8/ 10^6 pCEP-

RF cells and 19.6 ng IL-8/ 10^6 pCEP-2 cells and these values were diminished in the presence of 150 μM dicoumarol to 16.4 ng/ 10^6 pCEP-RF cells (48 % inhibition) and 8.6 ng/ 10^6 pCEP-2 cells (61 % inhibition) as shown in **Figure 6.9**. At a concentration of 38 μM, dicoumarol reduced pCEP-RF chemokine production further to 13.2 ng IL-8/ 10^6 cells, which is equivalent to 72 % inhibition; and this dose of dicoumarol inhibited IL-8 secretion by pCEP-2 cells by approximately 45 % to 11.4 ng/ 10^6 cells.

Similarly, TNF-α-induced IL-8 production by pCEP epithelial cells showed dose-dependent inhibition in those cells that had been treated with dicoumarol. TNF-α induced comparable levels of IL-8 in CF and non-CF pCEP cells (16.6 ng and 17.2 ng/ 10^6 cells, respectively), which were reduced by approximately 64 % of the induced in each case after exposure to 150 μM dicoumarol for 30 min. **Figure 6.10** illustrates that the same level of inhibition was observed in pCEP-RF cells at 75 μM dicoumarol, although TNF-α-stimulated pCEP-2 cells produced slightly more IL-8 at this inhibitor concentration (9.8 ng IL-8/ 10^6 cells, representing 54 % inhibition). The strongest effect that dicoumarol had on IL-8 secretion was in cells stimulated with *B. cepacia*. Bacterial stimulation of pCEP-2 cells with strain J2315 elicited 7.8 ng IL-8/ 10^6 cells. However, pre-treatment for 30 min with either 150 μM or 75 μM dicoumarol abrogated this induction completely, restoring pCEP-2 IL-8 secretion to resting cell levels (**Figure 6.11**). Similar inhibition was achieved with 38 μM dicoumarol pre-treatment, which limited IL-8 production in bacterial-stimulated pCEP-2 cells to 2.0 ng IL-8/ 10^6 cells.

6.3 Bacterial Activation of MAP Kinase in Airway Epithelial Cells.

To investigate the effects of bacterial stimulation on MAP kinase cascade activation, epithelial cells were grown in 35cm^2 petri dishes and stimulated with *B. cepacia* J2315 or with *P. aeruginosa* PAO1 for up to 1 h. Epithelial proteins in the whole cell lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were probed with monoclonal phospho-specific anti-ERK antibody and detected with chemiluminescence. Finally, blots were stripped and re-probed with polyclonal non-phospho-specific ERK antibody. The activation of ERK was quantified by densitometry. ERK-1 was not detected in all of the cell lines so that it was not possible to quantify changes in the level of activation of this protein. For this reason, only ERK-2 activation was examined.

6.3.1 Effects of *P. aeruginosa* on ERK-2 activity in airway epithelial cells.

All of the epithelial cell lines showed some change in activation (phosphorylation) of ERK-2 following stimulation with *P. aeruginosa* PAO1, although the kinetics varied amongst cell lines. Phosphorylated ERK-2 was detected in quiescent HTE cells but the strength of this signal was reduced at early time points (30 sec – 2 min) following bacterial stimulation (**Figure 6.12**). ERK-2 activity peaked approximately 5 min post stimulation, almost returning to unstimulated levels. Despite the observed reduction in phosphorylated ERK-2 in HTE cells after PAO1-stimulation, some degree of activity was detected throughout the experiment. In contrast, *P. aeruginosa* PAO1 elevated ERK-2 activation in CFTE cells (**Figure 6.13**), peaking at more than a 7-fold increase

by 2 min and this level of ERK-2 phosphorylation was still present 10 min after stimulation. A slight decrease was observed at the 5 min time point, but still ERK-2 phosphorylation was approximately 5 times its level in quiescent cells. ERK-2 activity dropped below unstimulated levels during the next 20 min before increasing again during the final 30 min of the experiment. Phosphorylated ERK-2 was present in resting pCEP cell lines and had begun to increase 5 min after PAO1-stimulation. This upregulation was sustained in pCEP-RF cells (**Figure 6.15**) reaching almost 4 times the level of phosphorylated ERK-2 that was observed in unstimulated controls, whilst ERK-2 activity declined after 15 min in pCEP-2 cells (**Figure 6.14**).

6.3.2 Effects of *B. cepacia* on ERK-2 activity in airway epithelial cells.

Within 30 sec of exposure to *B. cepacia* J2315, pCEP-RF cells had increased ERK-2 phosphorylation by 200 % (**Figure 6.17**), an effect that was mimicked at 5 min post bacterial stimulation. ERK-2 phosphorylation began to increase above unstimulated levels again at 10 min, until 30 min after initial stimulation, when pCEP-RF cells expressed twice as much phosphorylated ERK-2 as did quiescent cells. One hour after stimulation with J2315, the degree of ERK-2 phosphorylation in pCEP-RF cells had returned almost to constitutive levels. The initial increase in phosphorylated ERK-2 in *B. cepacia*-stimulated pCEP-2 cells displayed slightly different kinetics from pCEP-RF cells. ERK-2 phosphorylation had doubled 30 sec post stimulation, which rose to almost a 4-fold increase compared with quiescent cells by 2 min (**Figure 6.16**). Five minutes post stimulation some depletion of phosphorylated ERK-2 was noted but a second peak in expression was seen 10 min after initial stimulation of

pCEP-2 cells with *B. cepacia* J2315, when phosphorylated ERK-2 was more than three times its original concentration. ERK-2 phosphorylation decreased to less than double its original concentration by 15 min, and had returned almost to basal level 30 min post stimulation. ERK-2 activity began to increase again 60 min after initial stimulation of pCEP-2 cells with *B. cepacia* J2315.

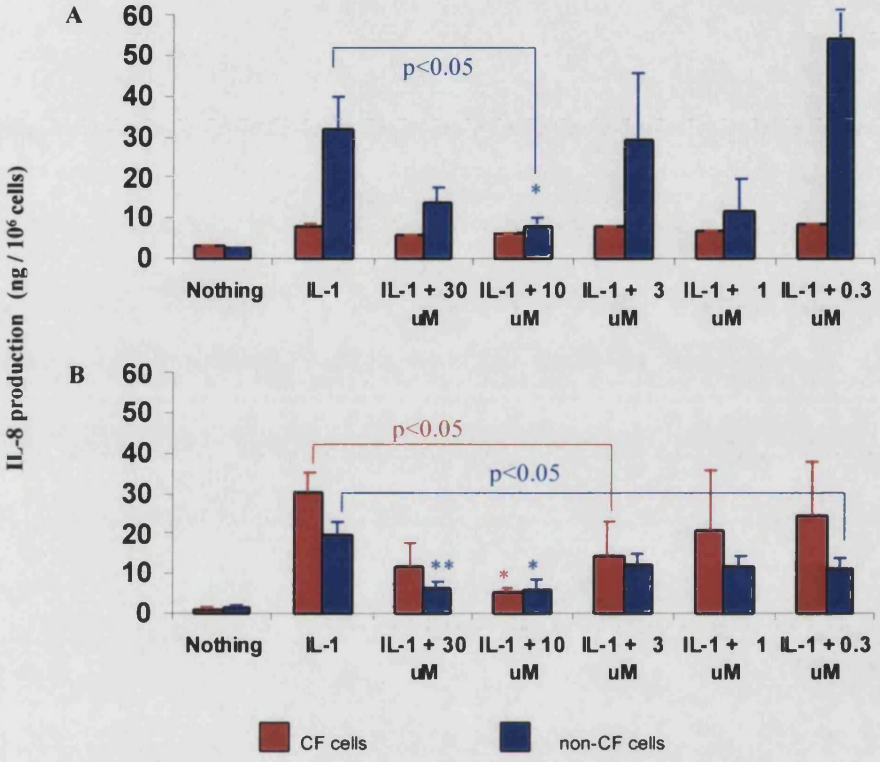


Figure 6.1. IL-8 production in epithelial cells after 24 h stimulation with 10 ng/ ml IL-1β following 1 h pre-treatment and 24 h antagonism with the p38 inhibitor SB-203580 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant (* $p<0.05$; ** $p<0.01$). "Nothing" n=26; "IL-1" n=11; all others n=3.

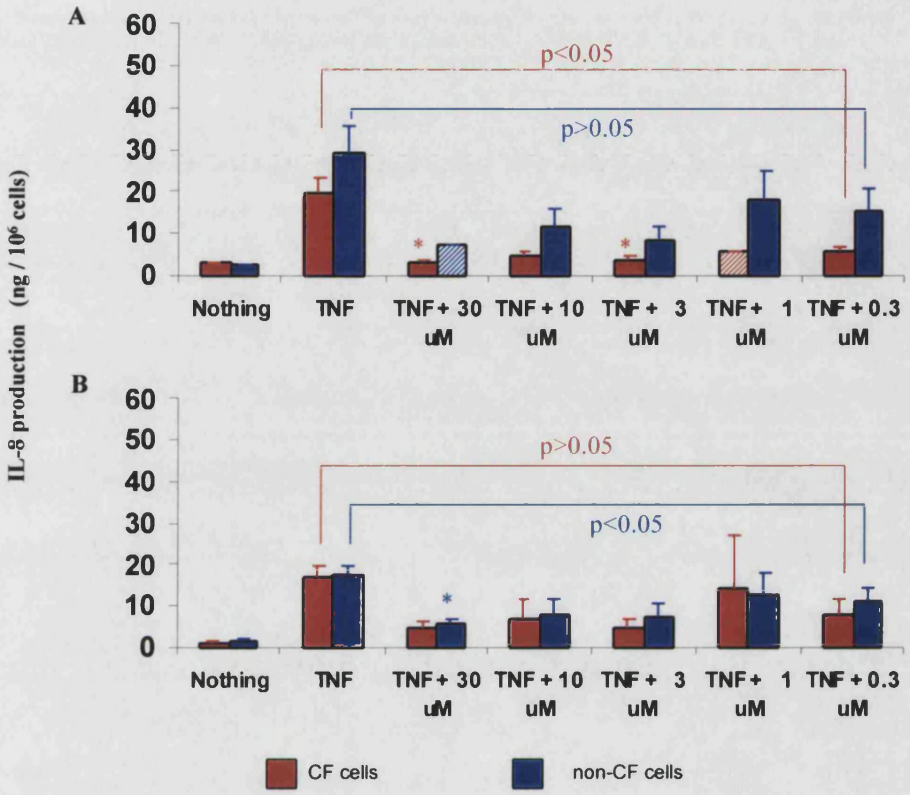


Figure 6.2. IL-8 production in epithelial cells after 24 h stimulation with 30 ng/ ml TNF-α following 1 h pre-treatment and 24 h antagonism with the p38 inhibitor SB-203580 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant (* $p<0.05$). "Nothing" n=26; "TNF" n=10; all others n=3; hatched bars indicate n<3.

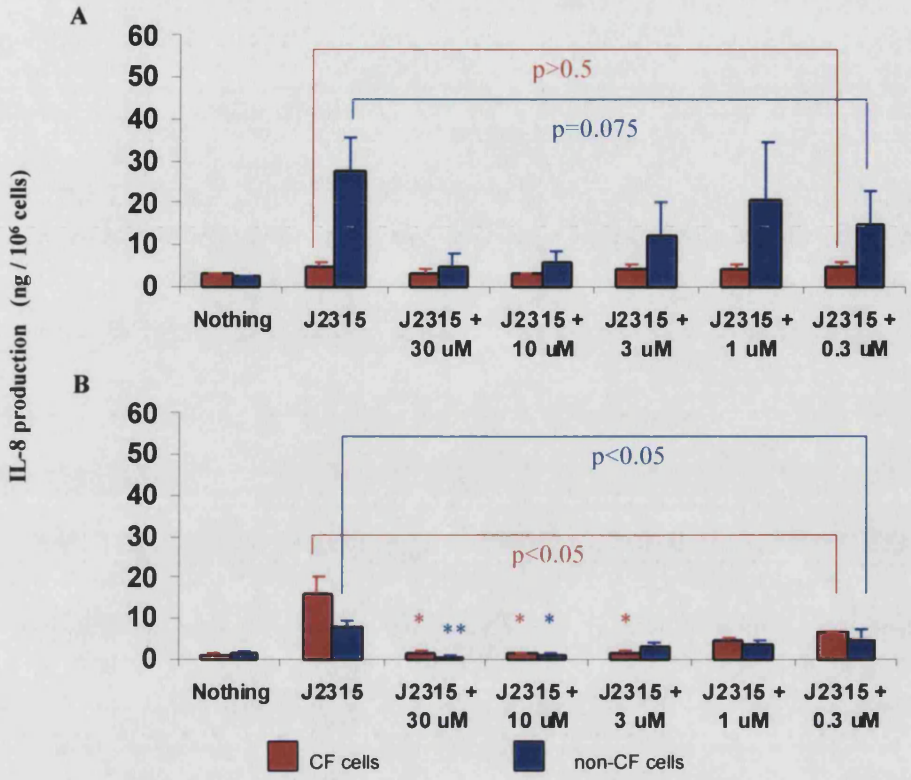


Figure 6.3. IL-8 production in epithelial cells after 24 h stimulation with *B. cepacia* J2315 (moi = 200) following 1 h pre-treatment and 24 h antagonism with the p38 inhibitor SB-203580 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant (* $p<0.05$; ** $p<0.01$). "Nothing" n=26; "J2315" n=6; all others n=3.

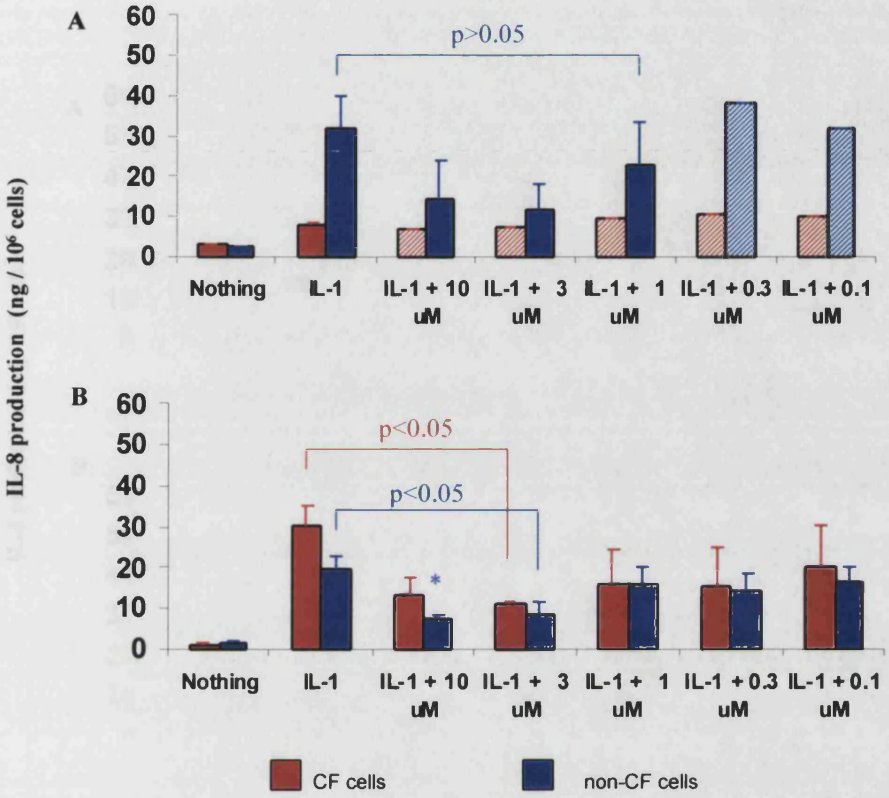


Figure 6.4. IL-8 production in epithelial cells after 24 h stimulation with 10 ng/ ml IL-1β following 1 h pre-treatment and 24 h antagonism with the ERK inhibitor PD-098059 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant. (* $p<0.05$). "Nothing" n=26; "IL-1" n=11; hatched bars indicate n<3; all others n=3.

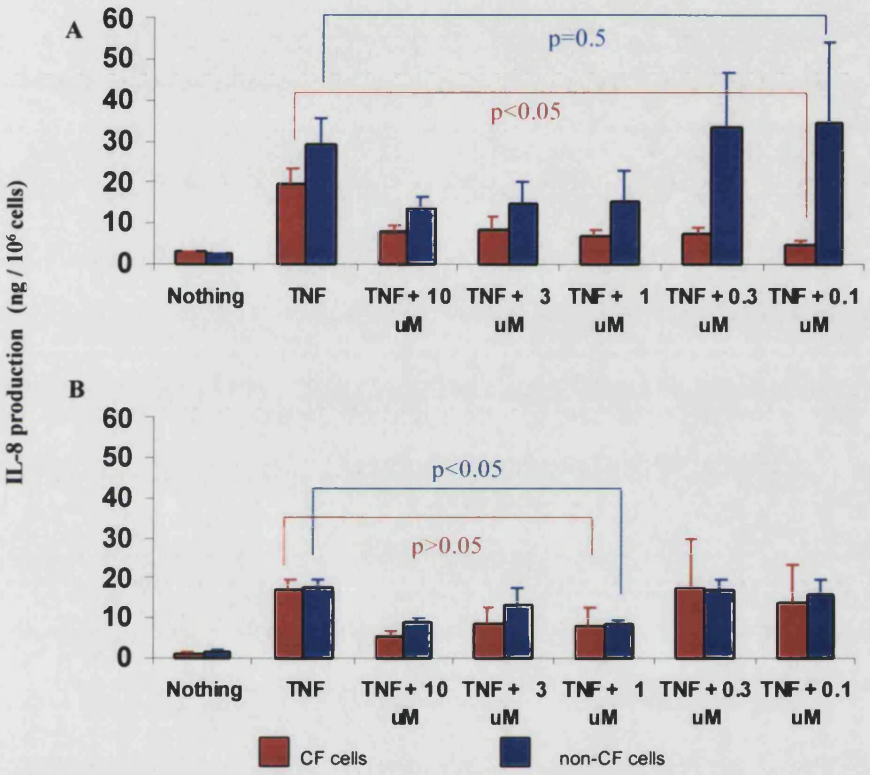


Figure 6.5. IL-8 production in epithelial cells after 24 h stimulation with 30 ng/ ml TNF-α following 1 h pre-treatment and 24 h antagonism with the ERK inhibitor PD-098059 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant. "Nothing" n=26; "TNF" n=10; all others n=3.

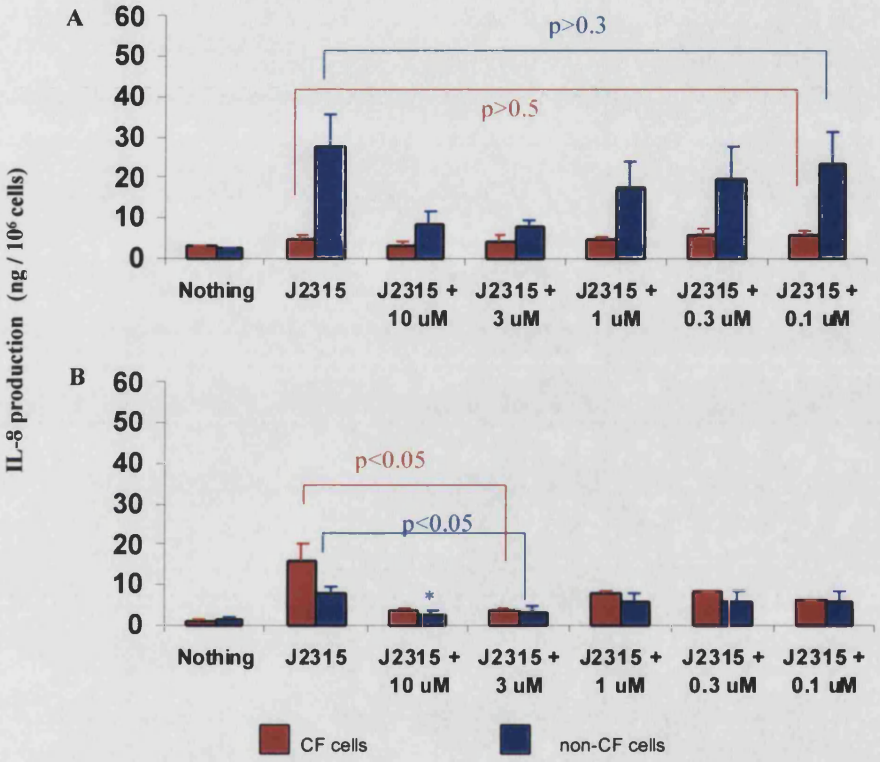


Figure 6.6. IL-8 production in epithelial cells after 24 h stimulation with *B. cepacia* J2315 (moi = 200) following 1 h pre-treatment and 24 h antagonism with the ERK inhibitor PD-098059 (mean + SEM). A: CFTE and HTE cells; B: pCEP-RF and pCEP-2 cells. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons where $p<0.05$ was considered significant. (* $p<0.05$). "Nothing" n=26; J2315 n=6; all others n=3.

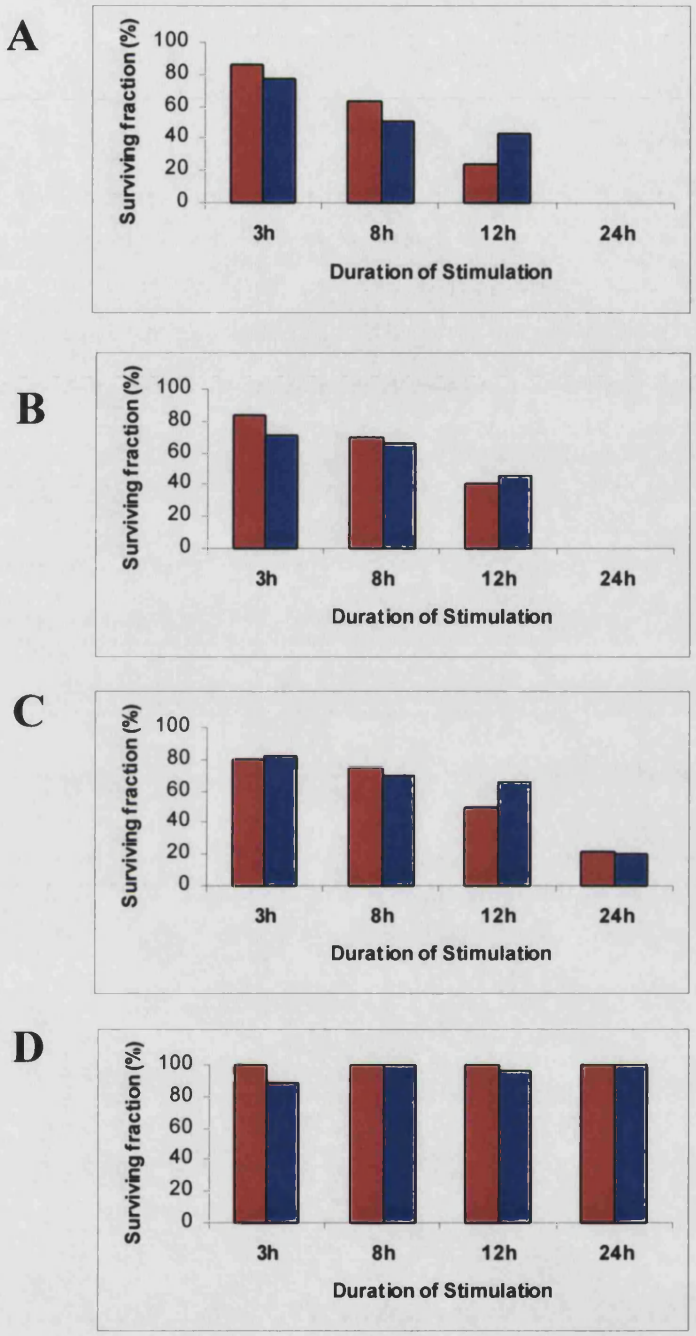


Figure 6.7. Toxicity of dicoumarol (24 h exposure) to pCEP airway epithelial cells. pCEP-RF (orange) and pCEP-2 (blue) epithelial cells were treated with dicoumarol and viability was assessed at intervals up to 24 h. A: 150 μM; B: 75 μM; C: 38 μM; D: vehicle control. (n=1).

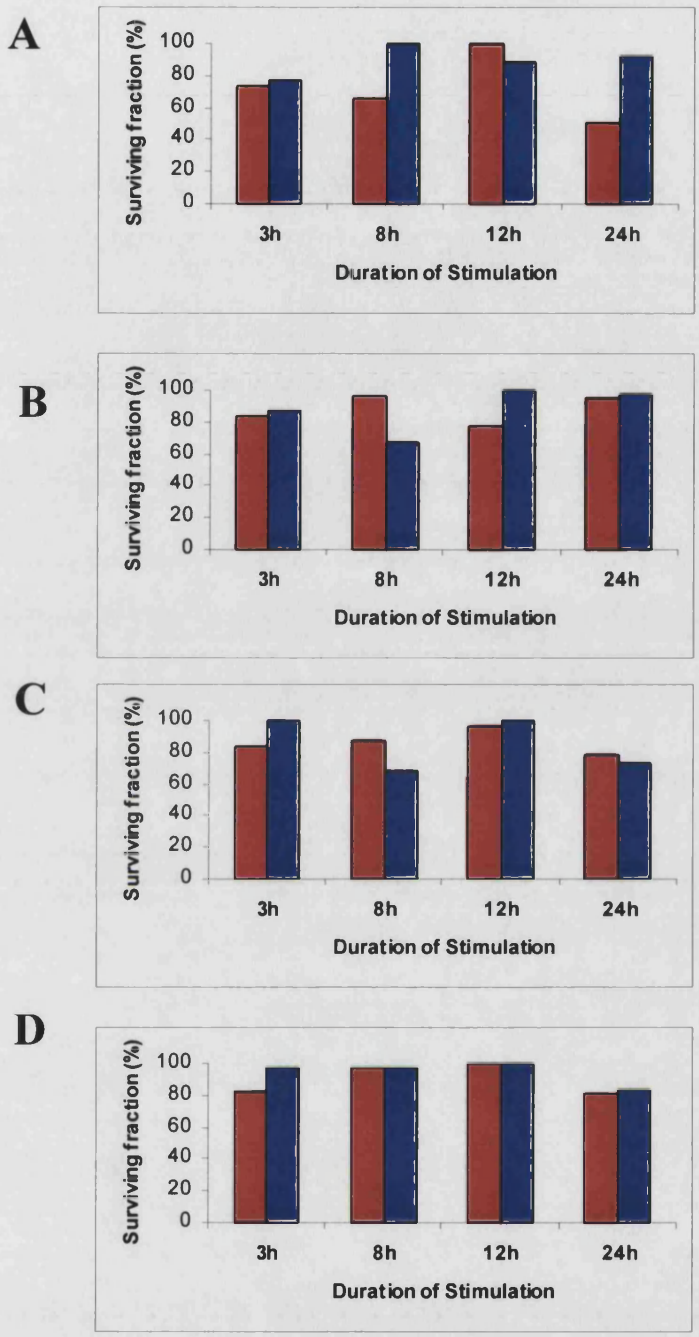


Figure 6.8. Toxicity of dicoumarol (30 min exposure) to pCEP airway epithelial cells. pCEP-RF (orange) and pCEP-2 (blue) epithelial cells were treated with dicoumarol for 30 min, washed and then viability was assessed. A: 150 μM; B: 75 μM; C: 38 μM; D: vehicle control. (n=1).

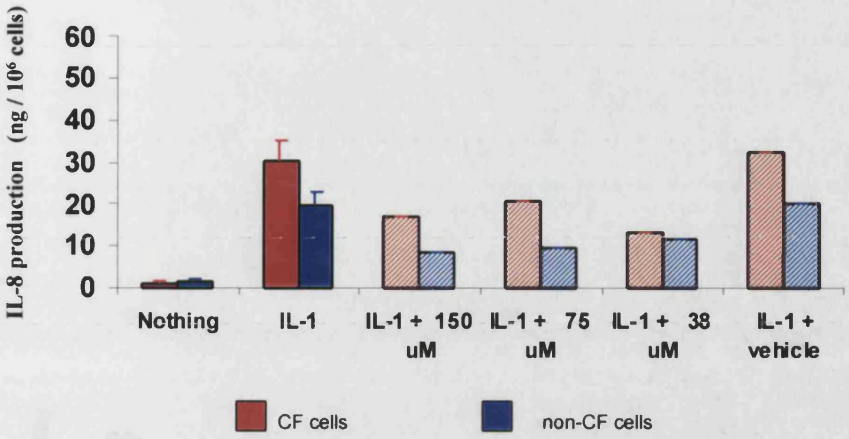


Figure 6.9. IL-8 production in pCEP epithelial cells after 24 h stimulation with 10 ng/ ml IL-1β following 30 min antagonism with dicoumarol (mean + SEM). "Nothing" n=26; "IL-1" n=11; hatched bars indicate n<3.

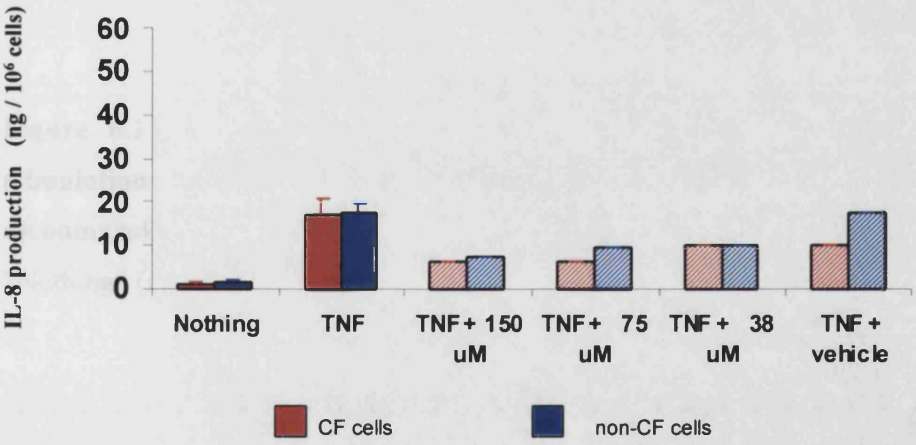


Figure 6.10. IL-8 production in pCEP epithelial cells after 24 h stimulation with 30 ng/ ml TNF-α following 30 min antagonism with dicoumarol (mean + SEM). "Nothing" n=26; "TNF" n=10; hatched bars indicate n<3.

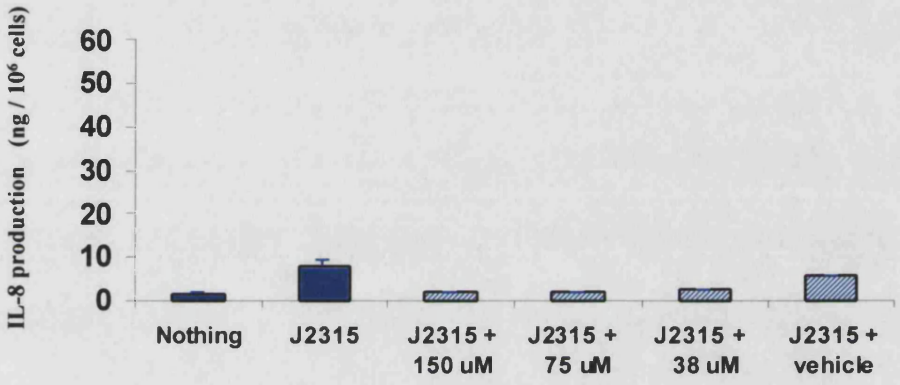


Figure 6.11. IL-8 production in pCEP-2 epithelial cells after 24 h stimulation with *B. cepacia* J2315 following 30 min antagonism with dicoumarol (mean + SEM). Hatched bars indicate n<3 [present in all except "Nothing" (n=26) and "J2315" (n=6)].

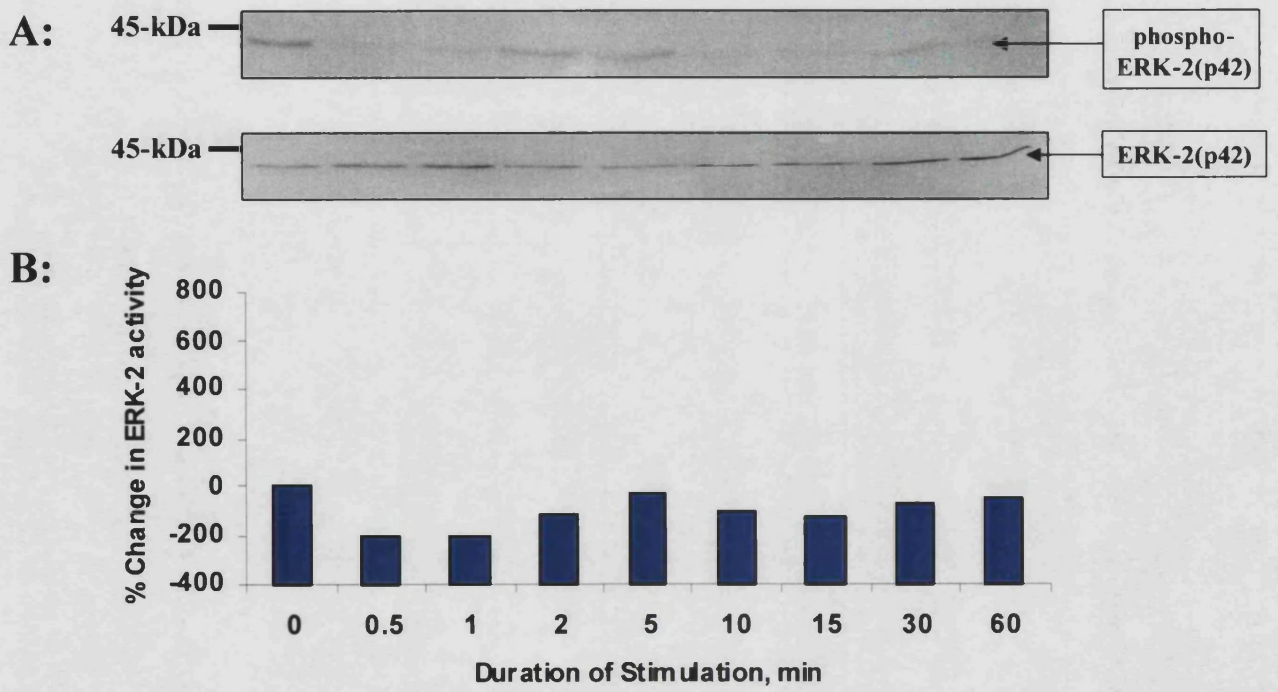


Figure 6.12 Phosphorylation (activation) of ERK in HTE cells following stimulation with *P. aeruginosa* PAO1.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-pan-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

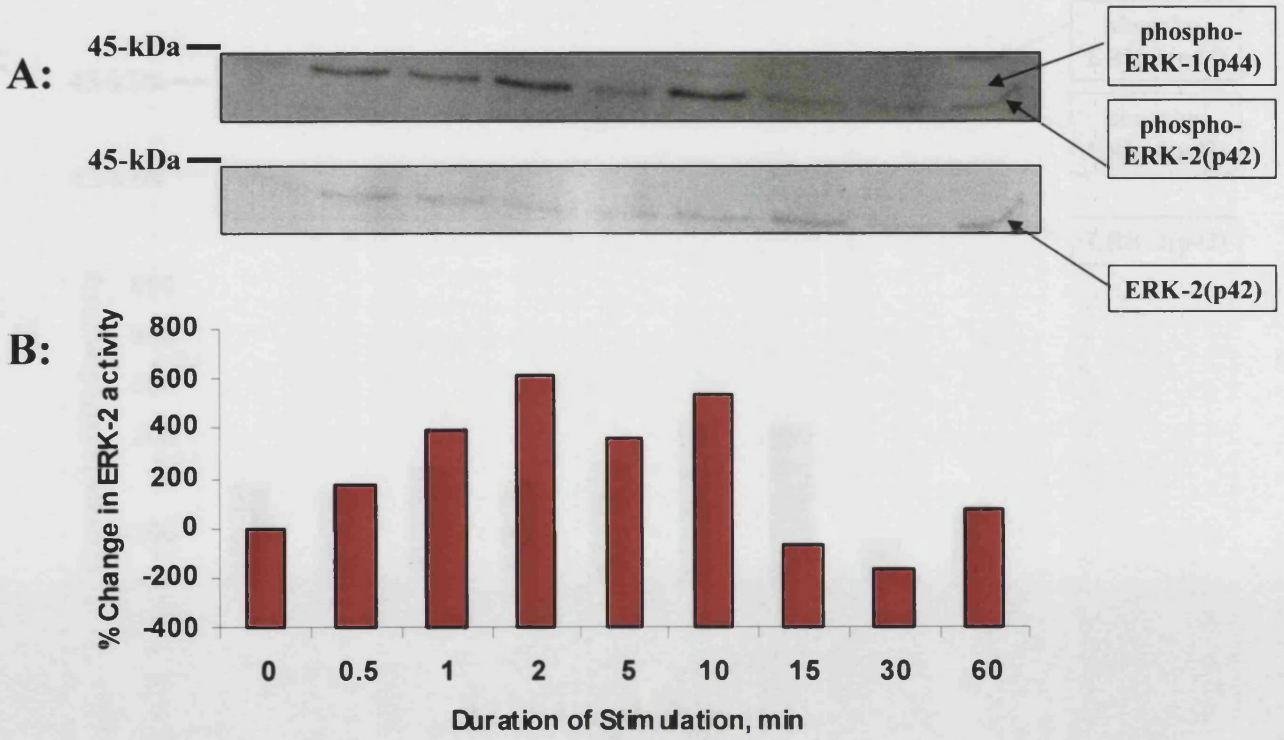


Figure 6.13 Phosphorylation (activation) of ERK in CFTE cells following stimulation with *P. aeruginosa* PAO1.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-pan-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

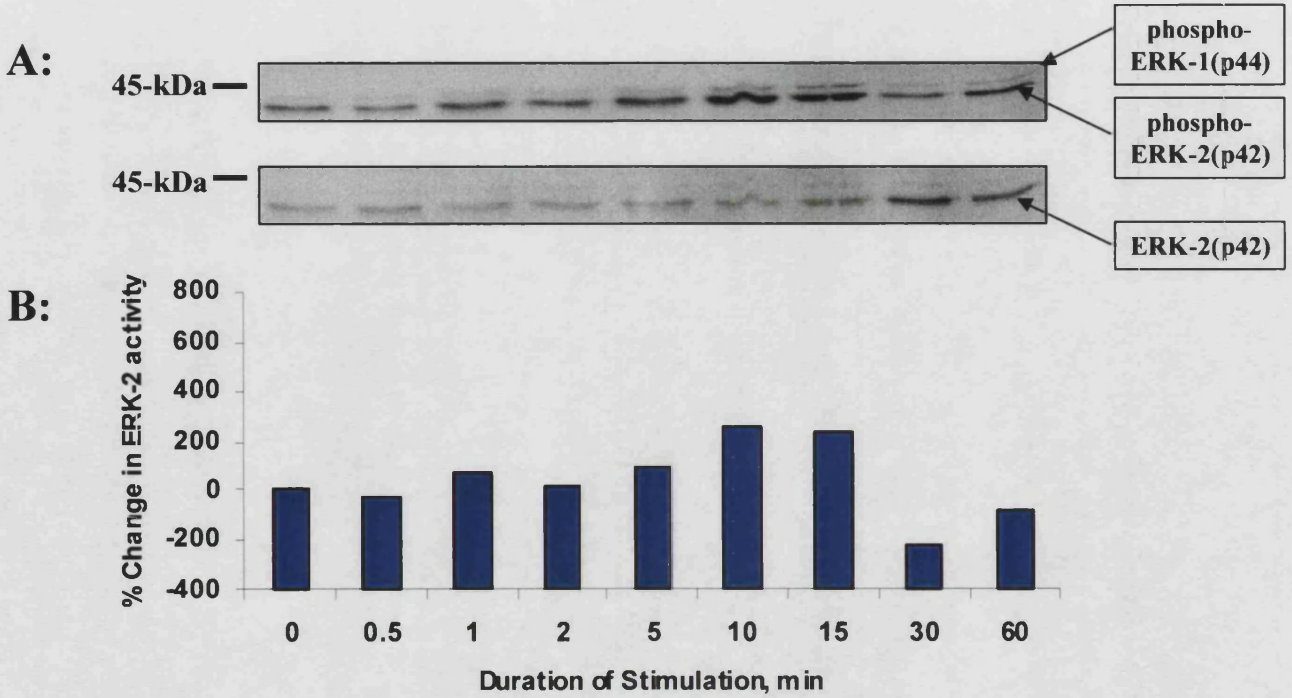


Figure 6.14 Phosphorylation (activation) of ERK in pCEP-2 cells following stimulation with *P. aeruginosa* PAO1.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-pan-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

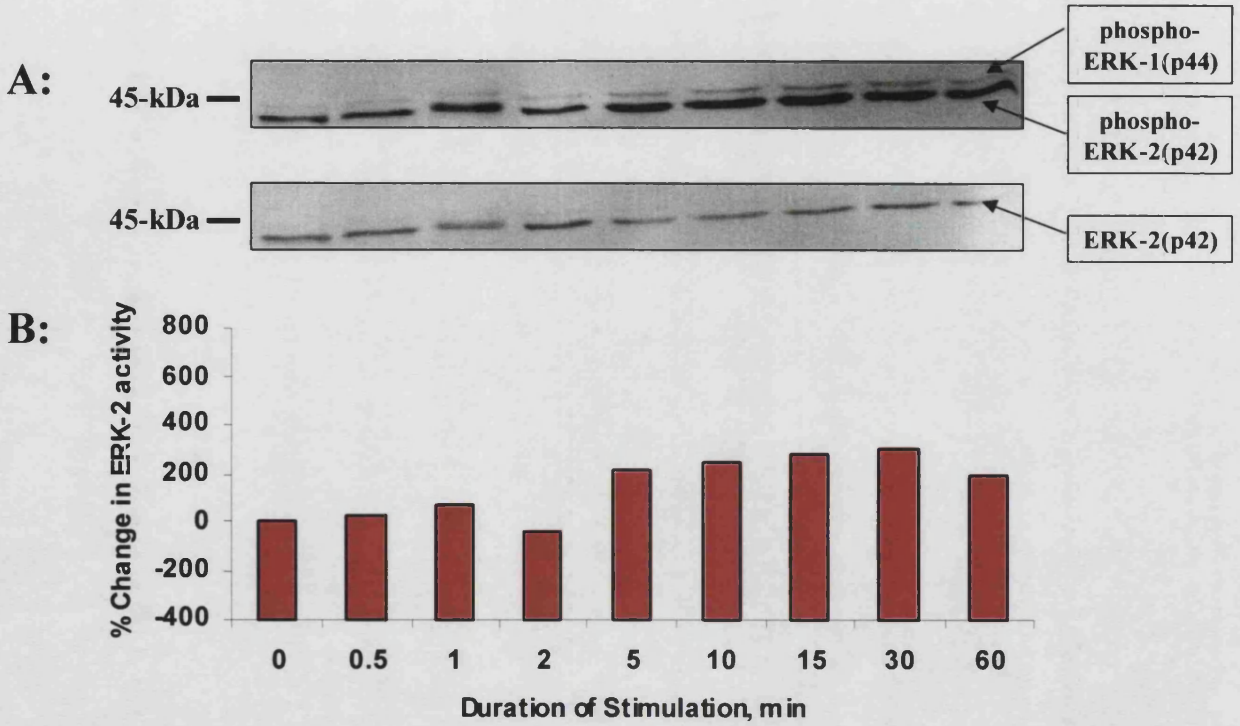


Figure 6.15 Phosphorylation (activation) of ERK in pCEP-RF cells following stimulation with *P. aeruginosa* PAO1.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

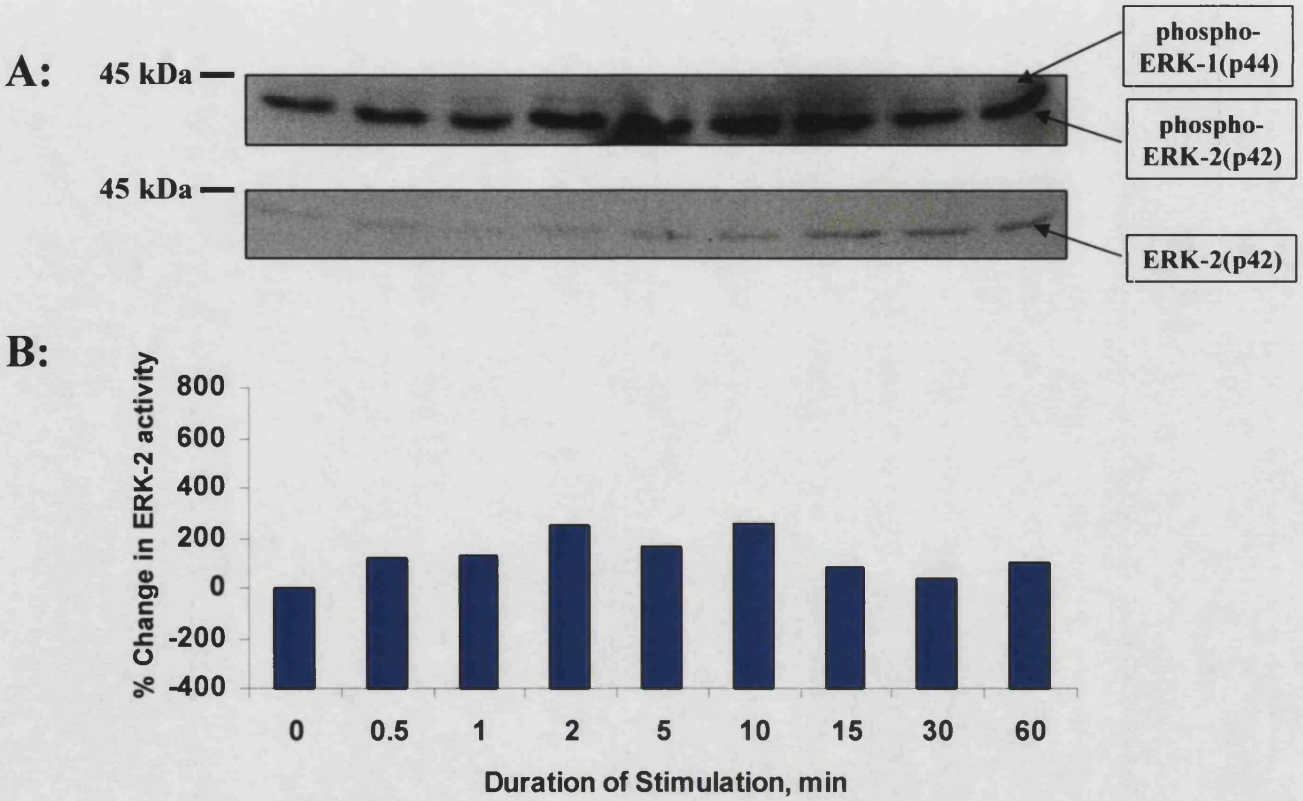


Figure 6.16 Phosphorylation (activation) of ERK in pCEP-2 cells following stimulation with *B. cepacia* J2315.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-pan-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

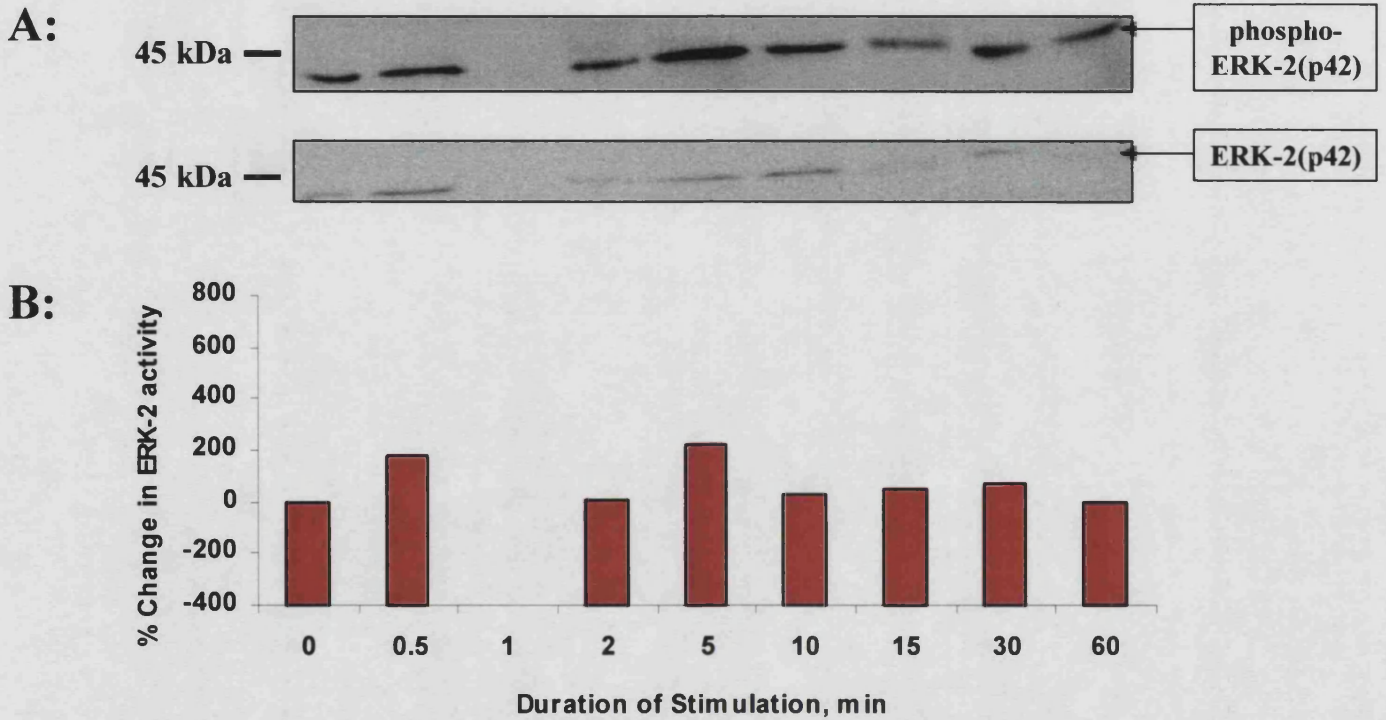


Figure 6.17 Phosphorylation (activation) of ERK in pCEP-RF cells following stimulation with *B. cepacia* J2315.

A: Western blot with anti-phospho-ERK antibody (top panel) and anti-pan-ERK antibody reprobe (bottom panel).

B: Histogram depicting % change in ERK activity following analysis of western blots using densitometry. (n=1).

6.4 Discussion.

Pulmonary disease in CF is characterised by an excessive influx of neutrophils into the lung, which is mediated primarily by the inflammatory cytokine IL-8 *(121;381)*. Several groups have observed elevated IL-8 production by CF respiratory epithelial cells in the absence of infection *(50;52;345;346)* and this situation is exacerbated both directly and indirectly by subsequent exposure to pathogens. The biochemical pathways leading to IL-8 have not been characterised completely, although some studies have identified a role for the transcription factor NFκB, which regulates the expression of many other mediators of immunity such as defensins, MHC-II and iNOS *(154;382-385)*. IL-1β and TNF-α as well as viral and bacterial products induce IL-8 secretion from a range of cell types, for example see references *(56;57;114;116;117;203;348;386-388)* and these stimuli may also activate members of the serine / threonine MAP kinase family *(362;389-393)* although there have been conflicting reports regarding the molecular route of these signals. NFκB is not the only transcription factor that regulates IL-8 gene expression *(376;394;395)*. Furthermore, whilst NFκB and MAP kinases can be activated independently of each other, they also share some distal signalling components as illustrated in **Figure 1.6**. In light of these facts, it seemed reasonable to investigate a possible involvement of MAP kinase cascades in the upregulation of IL-8 in airway epithelial cells and to identify potential differences in the regulation of IL-8 in CF versus normal cells.

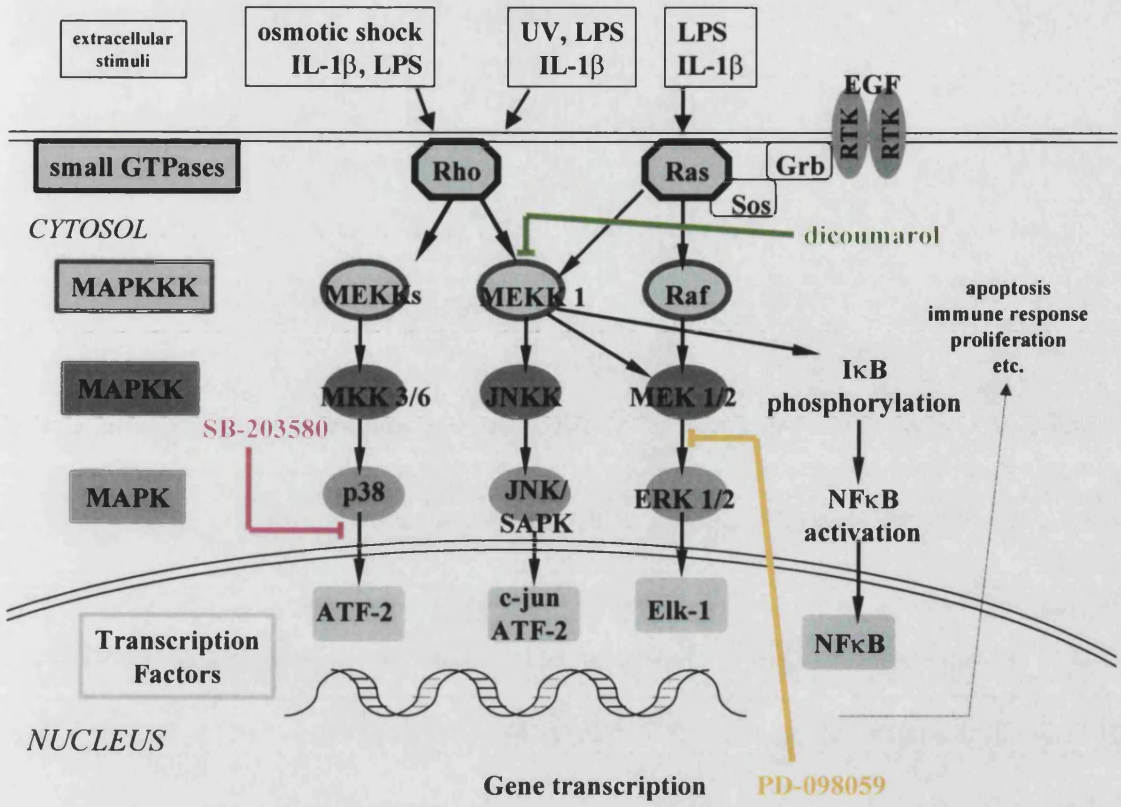


Figure 6.18 Inhibitors of MAP kinase signalling cascades. Dicoumarol prevents the activation of MEKK1, a membrane shuttle kinase that lies upstream of JNKK, MEK and also may regulate the NFκB pathway. SB-203580 is highly specific for p38 MAP kinase and prevents it from phosphorylating its downstream targets, such as the transcription factors ATF -2 and AP-1. PD-098059 antagonises MEK selectively and, until recently, this inhibitor was thought to be specific for ERK. However, a recent report has suggested that MEK may also phosphorylate JNK so that PD-098059 may inhibit JNK activation in some cell types.

There are three major MAP kinase families in mammals, namely ERK, p38 and JNK/SAPK. Numerous and varied unrelated extracellular cues can initiate intracellular signalling cascades that result in the phosphorylation of one or more of these MAP kinases, causing them to become activated. In turn, MAP kinases phosphorylate - and thus activate – transcription factors such as ATF-2

and Elk-1 *(373;396;397)*, that control the expression of subsets of genes. Specific and selective antagonists of MAP kinase pathways were assessed for their effects on basal and stimulated IL-8 production *in vitro* using an ELISA specific for IL-8. Although ERK and p38 inhibitors are available commercially, an equivalent antagonist of the JNK pathway has not been characterised. However, a recent study by Templeton *et al.* revealed that dicoumarol inhibited JNK MAP kinase *(380)* in CHO and HEK-293 cells. Dicoumarol is a derivative of coumarin, which has been used clinically as an oral anti-coagulant and this compound inhibits quinone reductase, an enzyme that lies downstream of the TNF receptor signalling complexes but upstream of the membrane shuttle kinase, MEKK1 (**Figure 6.18**). Therefore, dicoumarol was selected as an antagonist of the JNK signalling cascade.

Unfortunately, in their report Templeton *et al.* demonstrated that dicoumarol induced apoptosis in HeLa cells after 8 h, which was exacerbated in the presence of TNF-α *(380)* so that using a similar approach in this study would make it difficult to distinguish between any direct inhibitory effects of dicoumarol on IL-8 production, and an inhibition resulting from epithelial cell death. The toxicity of dicoumarol towards pCEP cells after 24 h was confirmed at a range of concentrations that inhibit quinone reductase (**Figure 6.7**). However, cell viability was compromised by less than a third in any pCEP cell population following exposure to dicoumarol for only 30 min (**Figure 6.8**). In general the most potent concentration of dicoumarol was 150 μM, which abrogated almost two thirds of cytokine-induced IL-8 production in pCEP-2 cells and three-quarters of IL-8 induced in pCEP-RF cells by TNF-α. However,

pCEP-RF cells stimulated with IL-1β and bacterial-stimulated pCEP-2 cells were inhibited most effectively by 38 μM dicoumarol: *B. cepacia*-induced IL-8 production was reduced below basal levels. It is unlikely that the inhibition resulted only, if at all, from epithelial cell death since inhibition values were greater than the corresponding cell death values at relevant concentrations of dicoumarol. Rather, it is probable that the effects of dicoumarol were caused by specific inhibition of one or more signal transduction pathways upstream of IL-8 induction in the respiratory epithelial cells. However, it should be stressed that these concentrations of dicoumarol are still relatively high and as such introduce a risk of non-specific inhibitory effects within the epithelial cells. The specific target of dicoumarol is MEKK1, a kinase that can activate either JNK MAP kinase or NFκB *(145;146;398)*, as shown in **Figures 1.7 and 6.18.** Thus, the observed effects of dicoumarol on stimulated epithelial IL-8 production may be mediated via inhibition of one or both of these signalling cascades. Although it has been reported that dicoumarol does not inhibit signalling pathways activated by mitogens *(380)*, some cross talk does exist between MEKK1 and MEK, which lies upstream of ERK MAP kinase (**Figure 1.6**), so the additional possibility that dicoumarol diminished IL-8 production because of ERK antagonism should not be discounted.

The role of p38 MAP kinase in epithelial IL-8 secretion was examined using the specific pharmacological inhibitor, SB-203580, a compound that blocks the kinase activity of p38 but has little or no detectable effect on ERK or JNK pathways *(316)*, as shown in **Figure 6.18.** Stimulated epithelial cells produced much less IL-8 if they had been pre-treated with SB-203580 than did

uninhibited controls and this antagonism was dose-dependent (**Figures 6.1-6.3**). In general, 10 μM SB-203580 was the most potent inhibitory concentration in IL-1β-stimulated cells and 30 μM SB-203580 inhibited TNF-α-induced IL-8 most effectively, reducing induced IL-8 by at least 70 % and by as much as 100 % in both CF and normal epithelial cells, with one exception. Since IL-1β had little stimulatory effect on IL-8 secretion from CFTE cells, an inhibitory effect of SB-203580 was less obvious in these cells. In contrast, TNF-α signalling pathways leading to the secretion of IL-8 were inhibited almost completely (98 %) in CFTE cells by this pharmacological antagonist, implying that p38 was an essential mediator of IL-8 induction in this cell line. In each cell line pair in the presence of TNF-α, antagonism of p38 MAP kinase had a greater effect in CF- than in normal cells (**Figure 6.2**). In agreement with other reports *(399-402)*, 75-80 % of TNF-α-induced IL-8 in all of the other cell lines could be abrogated with this MAP kinase antagonist, strengthening the idea that p38 is critically important in IL-8 upregulation following TNF-α-stimulation of the pulmonary epithelium. SB-203580 also reduced bacterial-induced IL-8 production and again the optimum antagonist concentration was 30 μM in most cases, reducing IL-8 production in *B. cepacia*-stimulated HTE cells by the greatest amount (**Figure 6.3**).

Finally, the role of ERK in epithelial cell IL-8 production was investigated and was found to vary according to both cell line and stimulus. Inhibiting ERK activation with PD-098059 eliminated approximately 50 %, 70 % and 80 % of induced IL-8 expression in HTE cells that had been stimulated with TNF-α, IL-1β or *B. cepacia*, respectively. The opposite trend was observed in CFTE cells,

however, in which ERK antagonism caused a striking reduction in the amount of IL-8 secreted in response to TNF-α; but this compound did not interfere as much in the levels of IL-8 induced by *B. cepacia* and had almost no effect in IL-1β-stimulated CFTE cells. These differences may be related to the distinct effects of each agonist on IL-8 induction in each cell population. Secretion of IL-8 was compromised by PD-098059 to a similar degree in both normal and CF pCEP cells stimulated with IL-1β, suggesting that the contribution of IL-1β signalling via ERK was the same in each case.

A greater effect of inhibiting ERK was seen in pCEP cells stimulated with *B. cepacia*, but again the degree of inhibition did not differ between cell populations expressing CF and normal phenotypes indicating that bacterial signalling to ERK may proceed in part via the TLR/ IL-1 pathway, perhaps through ligation of LPS receptors, but that another molecular route may also be involved in the activation of ERK by *B. cepacia* and that this alternative signalling pathway is active in CF and normal cells. Importantly, TNF-α-stimulated pCEP cells showed different responses towards PD-098059 according to their phenotype. In common with the first pair of cell lines (CFTE and HTE), IL-8 secretion was affected by ERK antagonism much more in pCEP-RF cells than in normal (pCEP-2) cells, in the presence of TNF-α. Again, it should be noted that TNF-α induced more IL-8 in cells with a CF phenotype than in normal cells and the elevation of TNF-α in the CF lung has been well documented *(103;403;404)*. These differences may relate to the efficiency of TNF-α to activate ERK or p38 in each case.

Table 6.1 summarises the relative inhibition of induced IL-8 in each cell line in the presence of different agonists. It can be seen from the data presented here that each of the inhibitors affected IL-8 induction to some extent in all of the cell lines, suggesting that all three MAP kinases and NFκB can mediate IL-8 production in airway epithelial cells. This apparent redundancy may provide alternative routes to regulate chemokine secretion in case one pathway becomes disrupted.

When used alone, both dicoumarol and SB-203580 completely ablated the induction of IL-8 by *B. cepacia* and this fact suggests that these signalling pathways are connected since dicoumarol does not affect p38 activity *(405)* and SB-203580 is a specific antagonist of p38 *(316)*. Hunninghake and co-workers reported that p38 is required for NFκB-dependent gene expression in endotoxin-stimulated monocytic cells, although p38 does not affect the activation of NFκB itself *(406)*. If NFκB-mediated transcription in tracheal epithelial cells stimulated with bacteria was also dependent upon p38 activity then inhibiting either of these signalling cascades would interfere with the expression of genes for proteins such as IL-8, consistent with the data presented here. Antagonism of the ERK pathway with PD-098059 also reduced the up-regulation of IL-8 although this inhibitor was not as efficacious as SB-203580 or dicoumarol. This finding suggests that ERK is responsible for at least some of the IL-8 production via an unknown transcription factor. Since MEKK1 is the molecular target of dicoumarol and some cross talk exists between this MAP3K and the ERK signalling cascade it might be expected that dicoumarol would also prevent the stimulation of IL-8 via ERK, despite the implications in Templeton's study that

ERK signalling remains unaffected by this inhibitor *(380)*. Taken together the effects of antagonising either ERK or p38 suggest that a common IL-8 transcription factor can be activated by both of these MAP kinases, and a possible candidate is AP-1 *(376;407-411)*.

The p38 MAP kinase appears to play the most important role in IL-8 secretion in both CF and non-CF cell lines regardless of the inflammatory signal. This conclusion is consistent with several published reports that describe the found p38 to play a central role in IL-8 production. It is also noteworthy that SB-203580 had the same or greater effect in epithelial cells expressing a CF phenotype than in normal cells, reflecting the relative importance of p38 in each cell type. Since one of the major extracellular stimuli for p38 signal transduction is osmotic stress *(138)*, it is perhaps not surprising that p38 might have a dominant role in CF cells, in which there is a pronounced disruption to electrolyte and fluid movement across the respiratory epithelium.

ERK activation was also measured indirectly in the epithelial cells following stimulation with either *B. cepacia* J2315 or *P. aeruginosa* PAO1. Whilst these studies give a good indication that ERK is enzymatically active, it should be appreciated that this approach measures only ERK phosphorylation, and does not measure the enzyme activity directly, such as could be achieved with an '*in vitro* kinase assay'. Even in the absence of bacterial stimulation, some phosphorylated (activated) ERK was observed in all of the cell lines, which may contribute to the production of IL-8 in unstimulated epithelial cells. Both bacterial strains activated ERK in pCEP-2 cells to a similar degree, peaking at

more than a 3-fold increase 10 min after stimulation compared with unstimulated cells, although *B. cepacia* activated ERK more rapidly than *P. aeruginosa* in this cell line (**Figure 6.19 A and B**). Approximately 20 min after stimulation with either bacterial strain ERK activity was reduced to basal levels, dephosphorylation continued for a further 10 min and began to rise again for the final 30 min of the experiment but did not quite return to unstimulated levels. In contrast, *P. aeruginosa* caused a delayed but sustained increase in phosphorylated ERK-2 in pCEP-RF cells, whilst *B. cepacia* elicited a biphasic and slightly lower elevation in the activity of this MAP kinase. Unexpectedly, *P. aeruginosa* induced dephosphorylation of ERK-2 in HTE cells with a rapid but transient 3-fold decrease in ERK-2 phosphorylation. The most dramatic change in ERK-2 activity was detected in *P. aeruginosa*-stimulated CFTE cells in which a rapid activation of ERK-2 was maintained for approximately 10 min, followed by an equally rapid dephosphorylation of ERK-2 below unstimulated levels. One hour after stimulation with *P. aeruginosa*, the level of phosphorylated ERK-2 in CFTE cells had returned to almost twice that in unstimulated control cells. It is particularly interesting to note that the pattern of ERK-2 activity in CFTE cells was the inverse of that seen in HTE cells (**Figure 6.19 C**).

The biphasic nature of ERK-2 activation within one hour of bacterial stimulation of respiratory epithelial cells has been reported previously following RSV infection *(354)* and may have a number of important physiological consequences. In addition to the involvement of ERK-2 in IL-8 production that has been demonstrated in this work and elsewhere *(354;412)*, ERK has also

been implicated in the upregulation of the *MUC1* gene for mucus production and in the prevention of immune cell apoptosis *(413-415)*, both of which are important during pulmonary infection in CF. However, it was not possible to identify any difference between CF and non-CF epithelial cells with regard to the activation of ERK-2.

Table 6.1 Summary of the effects of pharmacological inhibitors on IL-8 secretion in stimulated respiratory epithelial cells. Figures shown represent percentage inhibition of induced IL-8 production. Numbers in parentheses indicate the minimum concentration of inhibitor to mediate this inhibition.

INHIBITOR and STIMULUS	CELL LINE			
	CFTE	**HTE**	**pCEP-RF**	**pCEP-2**
Dicoumarol				
IL-1β	-	-	**75 %** (38 μM)	**61 %** (150 μM)
TNF-α	-	-	**64 %** (150 μM)	**64 %** (150μM)
B. cepacia	-	-	-	**100 %** (38 μM)
SB-203580				
IL-1β	No effect	**82 %** (10 μM)	**87 %** (10 μM)	**70 %** (10 μM)
TNF-α	**98 %** (30 μM)	**82 %** (30 μM)	**78 %** (30 μM)	**75 %** (30 μM)
B. cepacia	**92 %** (10 μM)	**90 %** (30 μM)	**99.6 %** (10 μM)	**100 %** (30 μM)
PD-098059				
IL-1β	No effect	**68 %** (3 μM)	**67 %** (10 μM)	**68 %** (10 μM)
TNF-α	**88 %** (0.01 μM)	**50 %** (10 μM)	**67 %** (10 μM)	**55 %** (10 μM)
B. cepacia	**71 %** (10 μM)	**79 %** (3 μM)	**84 %** (10 μM)	**85 %** (10 μM)

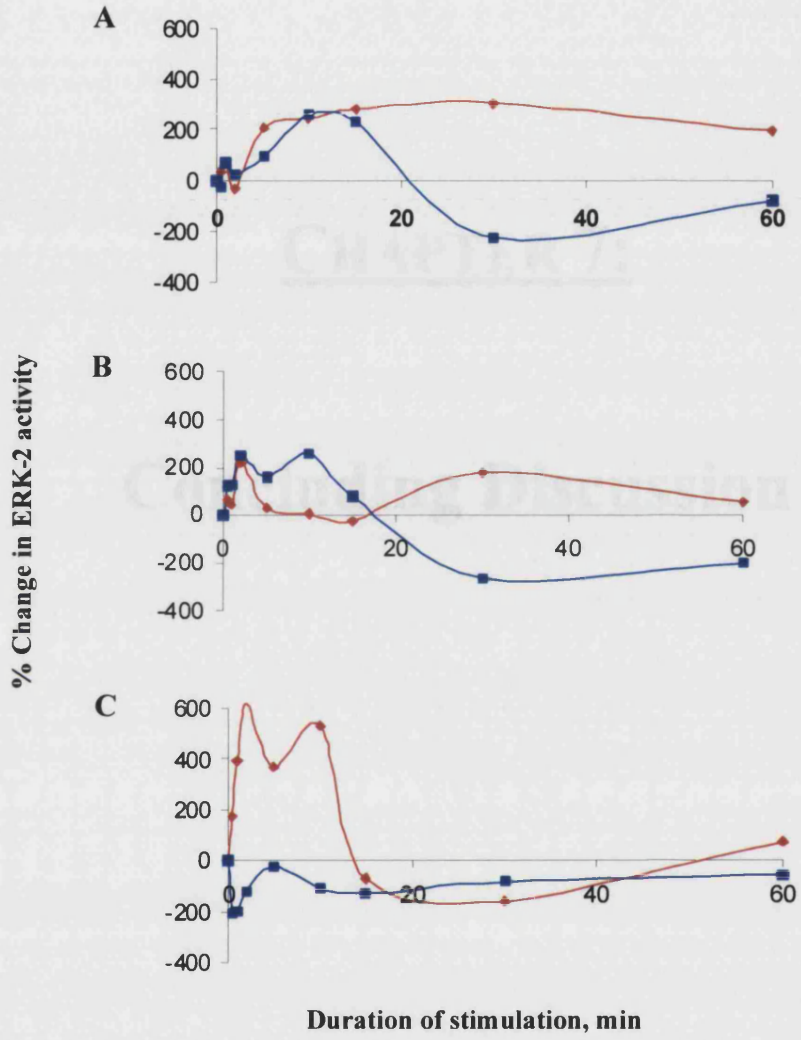


Figure 6.19 Kinetics of ERK-2 activation by bacteria in epithelial cells. A: pCEP-RF (red) and pCEP-2 (blue) cells stimulated with *P. aeruginosa* PAO1; B: pCEP cells stimulated with *B. cepacia* J2315; C: CFTE (red) and HTE (blue) cells stimulated with *P. aeruginosa* PAO1 (n=1).

CHAPTER 7:

Concluding Discussion

7.1 Concluding Remarks.

An inherently exaggerated inflammatory environment underscores the pathophysiology of the CF lung. Subsequent infection in most CF patients by highly adapted pathogens, which can resist the unfavourable conditions of the CF lung such as dehydration and nutrient limitation, exacerbates this situation further. However, whilst many theories have been proposed, the relationship between the primary genetic defect in the apical CFTR chloride channel, and the pre-disposition towards pulmonary infection and inflammation has not been determined satisfactorily and many questions remain unresolved. For example, it is curious that excessive inflammation and subsequent infection are restricted to the lung when multiple organs are affected by the CF gene mutation, and this caveat implies a failure of critical lung-specific innate defence mechanisms; and yet more curious is the fact that these infections are caused by so few species.

The present study was undertaken with two broad aims, namely to investigate whether CF pathogens are capable of regulating Cl^- channel activity in the lung and to evaluate the mechanisms by which bacteria induce epithelial cytokine production. Using a number of *in vitro* techniques, this study has examined both chloride channel activity and cytokine production in airway epithelial cells expressing either a CF- or a normal phenotype. In light of the data obtained in this and other studies, a potential connection between disrupted ion channel activity and the immune response within the CF lung is discussed.

Bacterial modulation of epithelial ion channels, including activation of CFTR by *E. coli (19;196;330)*, had been reported previously and in the latter example

CFTR stimulation promotes bacterial dissemination from the gut. It has been demonstrated in the work presented here that both *P. aeruginosa* and *B. cepacia* induce transepithelial chloride transport in airway epithelial cells, which may promote bacterial colonisation of the CF lung in a number of ways. For example, increased mucus viscosity, inactivation of salt-sensitive bactericidal agents in ASF *(69)*; or inactivation of neutrophils *(340)* would each facilitate survival of the pathogens in the lung. Alternatively, regulating electrolyte and fluid movements within the respiratory tract may allow the colonising bacteria to control the influx of antibacterial cells and biochemical mediators to the site of infection.

The mechanisms by which chloride transport processes were altered in the present work remain to be determined, but since no CFTR-dependent differences were identified, it is likely that another group of Cl^- channels was responsible for these observations, such as those regulated by Ca^{2+} (CaMCC) or ATP. *P. aeruginosa* binds via bacterial flagella to abundant asialo-GM_1 residues, which function as bacterial receptors, on CF airway epithelial cells and mobilises intracellular Ca^{2+} stores *(416)*, sometimes via ATP release *(417)*, with many potential physiological consequences, such as modulation of ciliary beating and mucus production. Furthermore, respiratory epithelial Ca^{2+} signalling may also be disrupted by *P. aeruginosa* pyocyanin *(320)*. Stimulation of Cl^- channels via bacterial-induced ATP release or intracellular waves of Ca^{2+} was not investigated by these research groups, but both remain enticing possibilities. It was observed in the current work that a structural,

rather than a secreted bacterial factor, induced the observed ion-channel modulation.

IL-8 production, as well as ion channel activity, was upregulated following stimulation with *P. aeruginosa* or *B. cepacia* although in contrast to results from the ion-channel studies, both isolated cells and supernatant from bacterial cultures elicited epithelial IL-8 secretion. Much of the pulmonary damage in CF is host-derived and results from an unusually high influx of neutrophils into the lung, recruited potently by IL-8 *(121)* that is secreted by epithelial cells and activated monocytic cells alike. As such, IL-8 is responsible for the initiation and maintenance of airway inflammation *(418)*. Subsequent release of ROS and proteolytic enzymes such as elastase from activated neutrophils is extremely damaging to the respiratory epithelium. Therefore, it is essential to elucidate upstream signalling pathways leading to IL-8 synthesis in order to identify potential targets for treatments to attenuate respiratory pathology. Increased pulmonary secretion of IL-1β and TNF-α during bacterial infection add to the upregulation of IL-8, and has been shown here and elsewhere to be mediated at least in part by MAP kinase cascades.

MAP kinase inhibitors afforded variable levels of protection against IL-8 induction by *B. cepacia*, which implicates MAP kinase signalling cascades in bacterial regulation of IL-8 and although an involvement of MAP kinases has been observed by other groups using *P. aeruginosa*, it is thought that this is a novel finding with respect to *B. cepacia*. Levels of phosphorylated ERK-2 were shown to be increased in the presence of *P. aeruginosa*, particularly in the

ΔF508 / ΔF508 cell line. Binding of *P. aeruginosa* via its flagella to asialo-GM_1 residues on the epithelial cell surface precipitates signalling via Ca^{2+}, which leads to the activation of ERK 1 / 2 *(321)*. ERK phosphorylates pp90rsk *(419)*, which in turn stimulates degradation of IκBα *(149)* and liberates NFκB from its cytosolic constraints. NFκB translocates into the nucleus and activates the transcription of many genes encoding proteins that are important in the host response to infection, such as IL-8, defensins, and iNOS. There is good evidence, presented both here and elsewhere, that ERK activation is pivotal in the induction of IL-8 by some agonists, but whether this MAP kinase always signals through NFκB has not been established. Moreover, ERK and IL-8 direct the immune system towards a T_H2 type response, which would encourage the intracellular survival of *B. cepacia* and seems to correlate with reduced clearance of *P. aeruginosa* as well as more rapid decline in CF lung function *(131;135)*.

Additional pathways of IL-8 regulation have been described in relation to other bacterial and viral respiratory pathogens including mediation via p38 MAP kinase, which has many downstream transcription factor targets. Interestingly, p38 seemed particularly important for IL-8 secretion in CF cells (Chapter 6). One of the major stimuli for p38 activation is osmotic stress *(143)* and in view of the characteristic osmotic dysregulation in CF, there is a very strong possibility that p38 is over-activated in CF. It is possible that hyper-activation of p38 contributes to elevated IL-8 production in quiescent epithelial cells. NFκB activation may also be mediated by p38 *(406)* and this MAP kinase has

been implicated specifically in further transcription of IL-1β *(420)*, and iNOS genes *(421)*.

During the course of this work, a non-toxic assay was developed by which dicoumarol could be used *in vitro* as a pharmacological tool to dissect intracellular signalling pathways. Dicoumarol is a specific inhibitor of quinone reductase *(380)*, an enzyme that gates redox activation of MEKK1. In turn, MEKK1 is a fundamental component of c-Jun and NFκB responses *(398)*, lying upstream of JNK MAP kinase and activating NFκB directly and independently of MAP kinase cascades *(146)*. Therefore, the observation that dicoumarol reduced stimulated IL-8 secretion from airway epithelial cells implicates one or both of JNK and NFκB in the regulation of expression of this chemokine. NFκB-regulated IL-8 secretion by epithelial tissue has been observed by several groups *(321;347;416;422)*, but the precise nature of events upstream of gene transcription have not been defined clearly. Results of experiments showing that dicoumarol inhibited IL-8 induction in airway epithelial cells (Chapter 6) suggest that JNK MAP kinase may also mediate IL-8 synthesis, although these effects could also have been caused by inhibition of NFκB. The specific roles of JNK and NFκB in IL-8 production in these epithelial cells were not assessed directly.

Work carried out in a number of laboratories has shown excessive activation of NFκB in naïve and stimulated cells expressing a range of CF phenotypes *(159;422;423)*. Both ER overload with mis-folded CFTR and a lack of functional CFTR expression have been implicated in this situation *(423;424)*.

More specifically, one group measured the level and type of IκBβ in CF versus normal cells and discovered that IκBβ is abnormally high in quiescent CF cells *(159)*. Furthermore, these researchers found that stimulated CF cells synthesised an unusually high proportion of the hypophosphorylated form of IκBβ, which remains in the nucleus and does not mask the NLS of NFκB. Thus NFκB activation is prolonged in CF compared with normal cells *(159)*. These observations might also account in part for the excessive production of IL-8 in quiescent and stimulated CF epithelial cells. Consensus binding sites for several other transcription factors have been located upstream of the IL-8 gene promoter so that NFκB may not be the sole regulator of expression of this gene. Indeed, AP-2 is also activated by *P. aeruginosa*, contributing to IL-8 expression *(416)*.

Bacterial factors that could be responsible for induction of this chemokine include LPS, flagella, autoinducer molecules or proteases. The endotoxic potential of *B. cepacia* LPS has been demonstrated in more than one study *(62;273;274;425)* and although *P. aeruginosa* LPS alone did not potentiate significant IL-8 release in the current work, other studies have shown endotoxicity to vary considerably amongst *P. aeruginosa* serotypes. Quorum sensing has been observed in both bacterial species and elevated levels of autoinducers have been detected in BAL from CF patients *(227)*. Furthermore, it was demonstrated very recently that the *P. aeruginosa* autoinducer molecule 3-O-C12-HSL elicits IL-8 secretion from host cells via ERK signalling to NFκB and AP-2 *(416)*. Signalling via ERK also elevates production of mucin *(419)*, which serves as a respiratory epithelial receptor for both *P. aeruginosa (189)*

and *B. cepacia (265)*. Synergistic interactions between *P. aeruginosa* and *B. cepacia* binding to respiratory epithelial cells have also been described *(267)*, although this concept was not investigated here. Manipulation of host cell signalling is a common and highly successful strategy in microbial pathogenicity, which includes many other examples such as ADP-ribosylation mediated by type III secretion system toxins *(244;248;250)*, and alteration of cytokine production to subvert the immune response *(426-428)*.

Currently, there is much interest surrounding the possibilities for treating CF at a very early age by correcting the underlying genetic defect. However, this approach would not be appropriate for all patients, such as those with established pulmonary inflammation and infection. Several studies have shown that the immune response in the CF lung is excessive and the regulation of this inappropriate inflammatory activity remains an important focus for clinical intervention in the treatment of CF. It is especially encouraging that treatment of rodent models of infection with anti-inflammatory agents such as ibuprofen or prednisolone ameliorated bronchiectasis without resulting in an increased bacterial load *(429-431)*. The reported hyperactivity of NFκB in the CF lung *(159;356;423;432)* makes this transcription factor an obvious target for therapeutic intervention to reduce bronchiectasis and improve lung function. It is intriguing to note that β-adrenergic agonists comprise a class of potent NFκB inhibitors *(433;434)*. These compounds have obvious beneficial effects regarding CFTR activation in the lung but β-adrenergic agonists may have additional clinical relevance to CF if they can be used *in vivo* to suppress excessive inflammatory responses via activation of NFκB. Natural dietary

products have also been used successfully in vitro to block NFκB activation *(435;436)* and many synthetic NFκB antagonists have been reported *(437)*.

In the wider context of CF, the data presented here have identified a number of potential targets that could be blocked to reduce inflammation, and these data corroborate other recent reports. Attempts to assess the relative contributions of each antimicrobial agent, defence strategy or signalling cascade must be considered in the context of the whole microenvironment within the lung since there is considerable intercommunication and redundancy in various aspects of respiratory immunology. Deletion of a single factor likely will be compensated by other local mechanisms although it is clear that respiratory infections persist in CF because of a combination of failed immunological mechanisms and disrupted signal transduction as well as the physiological adaptations of the CF microflora. The information presented here suggests that CF bacterial pathogens do indeed affect electrolyte transport and signal transduction in the lung and these disturbances could explain some but not all of these host-derived problems. Furthermore, such findings contribute to a more thorough molecular understanding of the host responses that operate in the respiratory tract, and how they are disturbed, which may permit the development of more effective treatments to prevent or alleviate symptoms associated with the progressive and ultimately fatal pulmonary disease that defines CF.

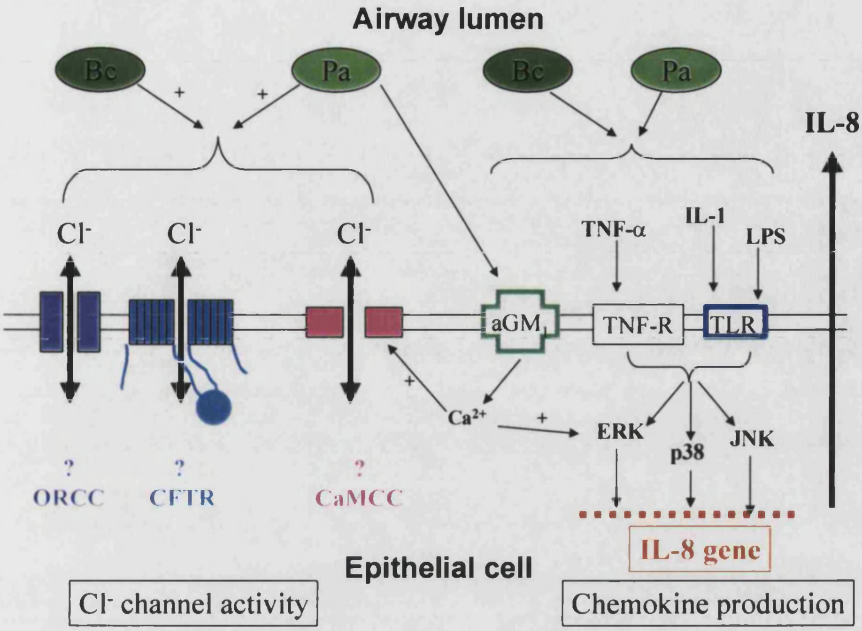


Figure 7.1 Summary of data presented in this thesis. Both *B. cepacia* (Bc) and *P. aeruginosa* (Pa) were found to activate chloride channel activity and IL-8 production in airway epithelial cells *in vitro*. The activated channels were not identified, although it is possible that CFTR is not implicated since Cl^- flux was measured in both CF and normal epithelial cells. Other possible candidates include the ORCC, which is also activated by CFTR; and CaMCC, which can be stimulated by waves of intracellular calcium. Similarly, both Bc and Pa induced IL-8 production by respiratory epithelial cells, independently of functional CFTR expression. In this study, the use of specific inhibitors of MAP kinases (ERK, p38 and JNK) suggested that each of these enzymes had a greater or lesser involvement in the up-regulation of IL-8 secretion following stimulation with bacteria or with the pro- inflammatory cytokines IL-1β or TNF-α, although the nature of the bacterial components and epithelial cell receptors responsible have not been determined in this work. It is possible that bacterial flagella binding to asialo-GM1 (aGM1) residues could mediate ERK activation and Cl^- channel activity. Another possibility is that LPS or secreted bacterial components activate IL-8 production via receptors similar to the TNF-receptor (TNF -R) or the TLR, since the kinetics of some bacterial responses were similar to those seen following stimulation with IL- 1β or TNF-α.

7.2 Future Work

Exciting observations made during the work presented in this study could be followed up in several ways. In particular, it would be interesting to investigate the following points:

- Characterise epithelial cell ion channel responses more closely in response to bacterial stimulation, using specific inhibitors of different chloride channels
- Employ specific antibodies against activated forms of JNK and p38 MAP kinases in experiments, with and without specific MAP kinase inhibitors, to clarify their respective roles in IL-8 production
- Establish the relative cellular effects of dicoumarol on NFκB and JNK signalling with respect to IL-8 production
- Examine NFκB activity in relation to *B. cepacia* stimulation and IL-8 production in these cell lines
- Investigate these responses in additional pairs of CF- and normal-epithelial cells to confirm current findings and how they relate to the functional expression of CFTR

APPENDIX I:

Solutions for Multiple Procedures

Table A1. Dulbecco's phosphate buffered saline (PBS) *(302)*

Component	Final concentration, g/L
$CaCl_2.2H_2O$	1.32
KCl	0.20
KH_2PO_4	0.20
$MgCl_2.6H_2O$	0.10
NaCl	8.0
Na_2HPO_4	1.15

Table A2. MEM non-essential amino acids *(304)*

Amino acid	Concentration in 100 x liquid
L-Alanine	0.89 g/L
L-Asparagine	1.32 g/L
L-Aspartic acid	1.33 g/L
L-Glutamine acid	1.47 g/L
Glycine	0.75 g/L
L-Proline	1.02 g/L
L-Serine	1.05 g/L

Table A3. M9 minimal salts

M9 component	Final concentration, g/L
$Na_2HPO_4.7H_2O$	**17.0**
KH_2PO_4	**4.0**
NaCl	**0.67**
NH_4Cl	**1.3**
D-glucose	**10.0**

Table A4. Tris-buffered saline (TBS), pH 7.4

Component	TBS
NaCl	9 g
Tris	1.211 g
Milli-Q	to 1 L

APPENDIX II:

Addresses of Suppliers

Alpha Laboratories Ltd., 40 Parham Drive, Eastleigh, Hampshire, SO50 4NU, U.K.

American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, U.S.A.

Amersham Pharmacia Biotech U.K. Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, U.K.

Anachem Ltd., 20 Charles Street, Luton, LU2 0EB, U.K.

BDH/Merck Ltd., Merck House, Poole, Dorset, BH15 1TD, U.K.

Becton Dickinson (U.K.) Ltd., Between Towns Road, Cowley, Oxfordshire, OX4 3LY, U.K.

Bibby Sterilin Ltd., Tilling Drive, Stone, Staffordshire, ST15 0SA, U.K.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, U.K.

BOC Gases, The Priestly Centre, 10 Priestly Road, Surrey Research Park, Guildford, Surrey, GU2 5XY, U.K.

Boehringer Mannheim – Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, U.K.

Buchi Labortechnik AG, Meierseggstrasse, CH-9230 Flawil, Switzerland

Calbiochem-Novabiochem (U.K.) Ltd., Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR

Ciba Corning Diagnostics Ltd., Colchester Road, Halstead, Essex, CO9 2DX, U.K.

Clonetics Corporation, 9620 Chesapeake Drive, San Diego, CA 92123, U.S.A.

Cohesion Technologies Inc., 2500 Faber Place, Palo Alto, CA 94303, U.S.A.

Costar U.K. Ltd., 10 The Valley Centre, Gordon Road, High Wycombe, Buckinghamshire, HP13 6EQ, U.K.

Dako Ltd., Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET, U.K.

Difco Laboratories, Detroit, MA, U.S.A. (supplied by Becton Dickinson (U.K.) Ltd.)

Dynatech Technologies – Dynex Technologies (U.K.), Action Court, Ashford Road, Ashford, Middlesex, TW15 1X B, U.K.

Elkay Laboratory Products (U.K.) Ltd., Unit 4, Marlborough Mews, Crockford Lane, Basingstoke, Hampshire, RG24 8NA, U.K.

Fisons (Fisher Scientific), Bishop Meadow Road, Loughborough, Leicestershire, LE11 0RG, U.K.

Gibco-Life Technologies: Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, U.K.

Grant Instruments (Cambridge) Ltd., Shepreth, Cambridgeshire, SG8 6GB, U.K.

Greiner Labortechnik Ltd., Station Road, Cam, Dursley, Gloucestershire, GL11 5NS, U.K.

Jouan Ltd., Merlin Way, Quarry Hill Road, Ilkeston, Derbyshire, DE7 4RA, U.K.

Millipore (U.K.) Ltd., The Boulevard, Blackmoor Lane, Watford, Hertfordshire, WD1 8YW, U.K.

Milton Roy Company (U.K.) Ltd., DMR House, Wokingham, Berkshire, RG11 2FD, U.K.

Molecular Probes Inc., 4849 Pitchford Avenue, Eugene, OR 97402-9165, U.S.A.

New Brunswick Scientific (U.K.) Ltd., Edison House, 163 Dixons Hill Road, North Mymms, Hartfield, Hertfordshire, AL9 7JE, U.K.

Nikon U.K. Ltd., Nikon House, 380 Richmond Road, Kingston upon Thames, Surrey, KT2 5PR, U.K.

Nunc, Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, U.K.

Perkin-Elmer Applied Biosystems (ABI), Kelvin Close, Birchwood Science Park North, Warrington, WA3 7PB, U.K.

PTI Inc., 1009 Lenox Drive, Suite 104, Lawrenceville, New Jersey 08648, U.S.A.

R&D Systems Europe Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxfordshire, OX14 3YS, U.K.

Sigma-Aldrich Chemical Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH, U.K.

Stuart, U.K. (division of Bibby Sterilin)

Techne (Cambridge) Ltd., Duxford, Cambridge, CB2 4PZ, U.K.

Tel-test, P.O. Box 1421, Friendswood, Texas 77546, U.S.A.

Wallac, Finland (supplied by Perkin Elmer Applied Biosystems)

Whatman International Ltd., Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent, ME16 0LJ, U.K.

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